NUMERICAL AND STRUCTURAL ABNORMALITIES OF CENTROSOMES IN ORAL CANCER AND PREMALIGNANCY

by

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ABSTRACT

Centrosomes play a critical role in cell division. Recent findings that both numerical and structural alterations to centrosomes occur in cancers support the possibility that such change may be a driving force for cancer development. However, little is known about centrosome alteration in oral cancers and premalignancies. The objective of this study was to assess the frequency of centrosome abnormalities in oral cancers and precancers utilizing immunofluorescence with antibodies to γ-tubulin, a well-characterized centrosomal component, and α/β-tubulin, the main component of centrioles. Fifty paraffin samples (13 oral cancers; 21 oral premalignancies; 16 nondysplastic controls) were used. The results showed a strong association of centrosome abnormalities and histology (cancer or presence/degree of dysplasia): size abnormalities: $P<0.001$; cluster formation: $P=0.001$, and more than 2 centrosomes in each cell: $P<0.001$. More than 90% of amplified centrosomes lacked centrioles. The results support a role for structural and numerical abnormalities of centrosomes in early carcinogenesis.
EXECUTIVE SUMMARY

**Background:** Early detection and management of oral premalignant lesions (OPLs) with a high risk of cancer development is the key to improving the poor 5-year survival rate of patients with oral squamous cells carcinoma (SCC). Histology is the current gold standard for both the diagnosis of malignancy and the assessment of cancer risk of premalignancy, each involving the evaluation of biopsies for the presence and degree of oral dysplasia. Although histology has good predictive value for high-grade lesions (most progress into cancer if left untreated), it has a limited value for low-grade lesions, which comprise the majority of OPLs. There is an urgent need to find a reliable risk predictor and to better understand oral carcinogenesis.

The process of oral carcinogenesis is still unclear. Genomic instability is a common phenomenon in human cancers including oral SCC. Most oral cancer cells carry about 60-90 chromosomes instead of the normal 46 chromosomes. In our laboratory, previous studies with Array Comparative Genomic Hybridization (array CGH) and fluorescence in-situ hybridization (FISH) analyses also showed an increase in DNA copy number for *FHIT, p16, TP53, cyclin D1, 7p11, 11q13, 3-21, 11q22.28-22.3 and 8q21-24* in oral cancer and OPLs. This is an early alteration that is strongly associated with oral carcinogenesis. The actual mechanism(s) responsible for such change is still unclear. Studies have shown a strong correlation between chromosome number changes, multipolar mitosis and centrosome abnormalities. Centrosome abnormalities could contribute to chromosome number changes through multipolar mitosis and cell cycle dysregulation including G1/S arrest, G2/M arrest and cytokinesis failure.
Numerical and structural alterations to centrosomes have been reported in several tumor types and could play a significant role in genomic instability and alteration to DNA copy number during carcinogenesis. There is currently little information available associating centrosomal abnormalities with human oral carcinogenesis and none available associating it with oral premalignancies.

**Objective:** The objective of this thesis was to determine whether there are centrosome alterations in oral carcinogenesis.

**Methods:** Fifty formalin-fixed paraffin-embedded oral tissue blocks (13 oral cancers; 21 oral premalignancies; 16 nondysplastic controls) were obtained from the British Columbia Oral Biopsy Service. An oral pathologist (Dr. Poh) evaluated all tissue blocks and confirmed the histological diagnosis according to World Health Organization criteria. Immunofluorescence analysis was used to detect centrosome abnormality in sections of tissue from each paraffin block with antibodies to γ-tubulin, a well-characterized centrosomal component, and α/β tubulin, the main component of centrioles.

**Results:** Numerical abnormality: The results showed that cells with more than 2 centrosomes (γ-tubulin signals) are significantly more frequent in high-grade dysplasia (average of 1.81% of cells in samples, \( P < 0.001 \)) and cancers (1.29%, \( P < 0.001 \)) compared with normal tissues (0.03%). Similar results were noticed when comparing high-grade dysplasia with low-grade dysplasia (1.81% vs. 0.69%, \( P = 0.004 \)). Cells with centrosomes in a cluster formation also appeared more frequently in high-grade dysplasia.
(1.42%, $P = 0.005$) and cancer tissues (1.18%, $P = 0.003$) than in normal tissues (0%). The chance of more than 1 type of number or shape changes of centrosomes increases with increasing histological severity ($P < 0.0001$). *Structural abnormality*: The average diameter of centrosomes increased significantly as histology progressed (0.46 ± 0.08μm in normal tissues, 0.45 ± 0.07μm in low-grade dysplasia, 0.54 ± 0.14μm in high-grade dysplasia, 0.71 ± 0.26μm in cancer tissues). By using 0.64μm as a cutoff line (the 95th percentile for normal cell diameters), the percentage of cells with abnormally enlarged centrosomes was found to be 3% in low-grade dysplasia, 23% in high-grade dysplasia and 55% in oral cancer tissues. Finally, more than 90% of amplified centrosomes in cells lacked centrioles and are considered acentriolar centrosomes.

**Conclusion**: This is the first report of a strong association of severity of numerical/structural centrosome abnormalities and oral cancer progression. The data support the possibility that centrosome abnormalities could play a significant role in human oral carcinogenesis.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKAP450</td>
<td>A-kinase anchoring protein 450</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division cycle</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
</tr>
<tr>
<td>CIS</td>
<td>Carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>DAPI</td>
<td>4′, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in-situ</em> hybridization</td>
</tr>
<tr>
<td>GCP(s)</td>
<td>Gamma-tubulin complex protein(s)</td>
</tr>
<tr>
<td>GI</td>
<td>Genomic instability</td>
</tr>
<tr>
<td>γTuRC(s)</td>
<td>Gamma-tubulin ring complex(es)</td>
</tr>
<tr>
<td>γTuSC(s)</td>
<td>Gamma-tubulin small complex(es)</td>
</tr>
<tr>
<td>HGD</td>
<td>High-grade dysplasia</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>hTERT-RPE</td>
<td>Human telomerase immortalized human retinal pigment epithelial</td>
</tr>
<tr>
<td>LGD</td>
<td>Low-grade dysplasia</td>
</tr>
<tr>
<td>OPL(s)</td>
<td>Oral premalignant lesion(s)</td>
</tr>
<tr>
<td>OSCC(s)</td>
<td>Oral squamous cell carcinoma(s)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid inhibitor</td>
</tr>
<tr>
<td>RR</td>
<td>Relative risk</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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I. INTRODUCTION

Oral squamous cell carcinoma (SCC) is the 8th most common malignancy worldwide and one of the top three most common cancers in south-central Asia, accounting for about 40% of all cancer cases in India (Mehrotra et al., 2003). It is believed to progress through sequential stages of pre-malignancies. Once oral SCC is formed, the prognosis is dismal. The 5-year survival rate for oral cancer is only slightly above 50% and this has not changed for the last 3 to 4 decades. Those who survive frequently have to endure severe cosmetic and functional compromise. One key factor in improving such dismal prognosis is early diagnosis. In the following sections, I will review oral cancer, precancer, and problems in their early diagnosis to show the urgent need for developing new tools to improve our ability to make early diagnosis; I will also review centrosome abnormalities and cancer development and will discuss the potential of using centrosome abnormalities as a new tool in the early diagnosis of high-risk oral premalignant lesions.

I.1. ORAL CANCER

I.1.1. Incidence and epidemiology

The 2002 annual report of the World Health Organization (WHO) showed that developing countries accounted for two-thirds of the 274,300 new oral cancer cases worldwide (Parkin et al., 2005). In 2006, over 3,100 new cases were expected in Canada and almost 31,000 new cases were expected in the United States (Canadian Cancer
Society, Canada, 2006; National Cancer Institute, 2006). In addition to geographical variances, the incidence rates of oral cancer show significant differences in gender. Men currently account for two-thirds of oral cancer cases. However, the ratio of male to female cases has dropped from 6:1 in 1950 to 2:1 in 1997 due to increasing smoking rates among women (CDC, 1998). Like most malignant tumors, oral cancer is age-related, with more than 95% of patients diagnosed after age 40 (CDC, 1998). With an aging population, incidence rates of oral cancer have been increasing sharply in many countries throughout the world, including Denmark, Scotland, Germany, eastern and central Europe, Australia, New Zealand, Japan and the US. This increase will continue for the next twenty years as forecasted by the World Health Organization (WHO) (Stewart et al., 2003).

I.1.2. Risk factors and etiologies

I.1.2.1. Tobacco smoking

Studies consistently show tobacco smoking playing a crucial role in oral carcinogenesis. Almost 80% of oral cancer patients were smokers at diagnosis. The average relative risk (RR) for tobacco smoking is about 4 to 5 compared to non-smokers (Vineis et al., 2004). There is no threshold for smoking that is considered to be without cancer risk. A strong dose-response effect exists among current smokers and ex-smokers (Castellsague et al., 2004; Rosenquist et al., 2005). The risk of oral cancer drops significantly after smoking ceases for 1-3 years. The risk continues to drop and approaches non-smoker risk levels
after smoking ceases for 10-17 years (Franco et al., 1989; De Stefani et al., 1998; Castellsague et al., 2004). The use of black tobacco cigarettes (RR = 3.3) results in higher risks compared to the use of blond tobacco cigarettes and hand-rolled cigarettes (De Stefani et al., 1998; Castellsague et al., 2004).

### 1.1.2.2. Other tobacco usage

Higher risk of oral cancer is also noticed among cigar (RR = 4.0) and pipe smokers compared to non-smokers (RR = 13.9). Bidi smokers have a 2-5-fold higher risk of oral cancer formation (Franco et al., 1989; Shapiro et al., 2000; Rahman et al., 2003). Oral smokeless tobacco is a common habit in some cultures and may be a major part of daily life. In Saudi Arabia, oral cancer incidence is associated with smokeless tobacco (shamma) use (Allard et al., 1999). Betel leaf or betel quid (betel leave with areca nut) is very popular in India, South Africa and South East Asia and is a strong risk factor in oral carcinogenesis. Betel quid chewing increases oral cancer risk 17-fold and is specifically associated with the elevation of buccal cancer (location where quid is placed in mouth) in India (Chen et al., 2002; Mehrotra et al., 2003). Higher intensity and longer duration of betel quid chewing increases the relative risk of death in patients diagnosed with OSCC in the buccal mucosa. The relative risk of death is 31.4 times higher in heavy users (> 30 years, > 30 quids per day, started before age 20) compared to mild users (< 10 years, < 15 quids per day, started after age 20) in Taiwan (Lee et al., 2005). In Sudan, a retrospective study shows that toomback dipping increases oral cancer risk by 3.9 to 4.3 times (Idris et al., 1995). Snuff inhalation (another form of tobacco usage) does not influence risk of
oral cavity cancer but instead is associated with cancers in the nasopharyngeal area (Nandakumar et al., 1990; Rosenquist et al., 2005).

### I.1.2.3. Alcohol

Alcohol use helps promote smoking-induced oral cancer development. One possible mechanism for this effect is that alcohol increases the penetration of carcinogens from tobacco through the oral mucosa. The relative risk is 50 times higher for heavy smokers (≥ 21 cigarettes per day) with high alcohol consumption (≥ 6 drinks per day) (Boyle et al., 2003; Castellsague et al., 2004). However, recent studies show that alcohol alone could also promote oral cancer development. The mechanism is still unknown the possible events include: (1) carcinogenic effects of acetaldehyde, impurities or contaminants in alcoholic drinks; (2) increased cell proliferation; and (3) chronic infection due to the accumulation of pathogenic microorganisms (Boyle et al., 2003; Riedel et al., 2005; Boffetta et al., 2006).

Although there is no clear threshold, a dose-effect relationship between drinking and oral cancer risk does exist. Both higher intensity and longer duration of drinking raise risks. In 2004, a meta-analysis revealed that drinking alone without smoking increases relative risks of oral cancer. The relative risks increase from 1.86 for mild drinkers (25g/day), to 3.11 for moderate drinkers (50g/day) and 6.45 for heavy drinkers (100g/day) (Corrao et al., 2004). The odds ratio significantly increases to 2.49 after 20 years of drinking and then doubles after 50 years of drinking. The cancer risk significantly drops after drinking
ceases for 3 years and will approach the level of non-drinkers 14 years after cessation (Castellsague et al., 2004).

1.1.2.4. Human papillomavirus infection

High-risk human papillomavirus (HPV) infection has been associated with increased risk of oral cancer. The odds ratio for HPV infection with high-risk strains is consistently elevated but varies broadly in different studies ranging from 2.6 to 63 times higher (Chen et al., 2002; Smith et al., 2004; Hansson et al., 2005). On average, high-risk HPV is present in 20-30% of oral cancers. (Sugerman et al., 1997; Castle, 2004). HPV 16 is detected more frequently than other HPV types. HPV 16 with or without a combination of other HPV types accounts for up to 81% of oral cancer with HPV infection (Smith et al., 2004; Hansson et al., 2005).

1.1.2.5. Age

Oral cancer is an age-related disease. The incidence rates go up dramatically after age 40. The average age at diagnosis is around 60 (CDC, 1998). In India, the age-specific incidence rates of men are 4.8 at age 35-44, 27.2 at age 55-64 and 59.7 at age over 75. And the age-specific incidence rates of women are 1.9 at age 35-44, 16.8 at age 55-64 and 25.2 at age over 75 (Sen et al., 2002).
I.1.2.6. **Other factors**

Ultraviolet B radiation exposure is positively associated with lip cancer but not with cancers of other parts of the oral cavity. The other possible risk factors include immunosuppression (Preciado *et al.*, 2002), folate deficiency (Pelucchi *et al.*, 2003), low socioeconomic status, poor oral hygiene and poorly fitting dentures (Hashibe *et al.*, 2003). Further studies are needed to determine the importance of these risk factors.

I.1.3. **Clinical features**

The signs and clinical features of oral cancers are varied on the lip and intra-oral areas. Lip cancer often starts on one side of the midline with presentations of shallow ulceration, thickening or crusting. Lip cancer is more commonly found on the lower lip than the upper lip. The spread to submental lymph nodes is slow (Coulthard *et al.*, 2003).

The most common sites of intra-oral cancers are the floor of the tongue, ventral tongue and lateral anterior tongue in North America (Bsoul *et al.*, 2005). Recently, increased numbers of OSCCs were found on the gingival (Khan *et al.*, 2005), periodontium (Levi *et al.*, 2005), and peri-implant areas (Block *et al.*, 2001; Czerninski *et al.*, 2006). Extensive use of smokeless tobacco and betel leaf (or betel quid) chewing among the Indian population shifts their most common oral cancer sites to the tongue (44%) and the buccal mucosa (19%) (Mehrotra *et al.*, 2003). Intra-oral cancers are often symptomless during the early stages but cause clinical changes such as bleeding, loss of teeth,
paresthesia, anaesthesia or development of a neck mass as the cancer progresses. The typical presentation is a hard, fixed necrotic ulcer with raised margins and color changes (red or red with white hues). However, the cancer might be present as a persistent mass, nodules, patches, crater-like ulcer, punched-out ulcers, polyps or granular lesions. The tumors spread distantly through the submandibular, cervical and jugular lymphatic systems (Neville et al., 2002; Coulthard et al., 2003; Bsoul et al., 2005).

1.1.4. Diagnosis

Collecting historical profiles is the first step in identifying high-risk patients. The basic components of a historical profile are medical, family and social histories. The second step is an oral screening examination to identify any abnormal changes of tissues and structures. A histopathological report from the biopsy is the gold standard in diagnosing oral cancer. A biopsy could be obtained by either excisional or incisional methods. Excisional biopsies are performed on relatively small lesions by removing the whole lesions with surrounding normal tissues. Incisional biopsies are performed on large lesions. Currently, clinicians tend to use toluidine blue vital staining (Chen et al., 2006), autofluorescence (Svistun et al., 2004), or chemiluminescence (Ram et al., 2005) to improve the diagnostic yields. However, these tools have not been validated and may not be cost-effective in diagnosing oral cancers. After the diagnosis of oral cancer, computer tomography (CT) and/or magnetic resonance imaging (MRI) will help in evaluating the extent of the cancer and in determining future treatment plans (Bsoul et al., 2005).
I.1.5. Treatment

The three current principal therapeutic methods are surgery, chemotherapy and radiotherapy. Treatment could be one or a combination of the three principal treatments based on the stage of the cancer. The T (size of tumor) N (involvement of lymph nodes) M (presence of distant metastasis) stage system is widely used in staging the disease, planning treatments and predicting the prognosis.

When cancers are still at the early stages, either surgery or radiotherapy yields similar outcomes. Once the cancer reaches the late stages, use of a combination of treatments is preferred to improve local regional control or survival rates. The combination of radiotherapy and chemotherapy after surgery does improve the local regional control or survival rates (Bernier et al., 2004; Cooper et al., 2004). To improve overall life quality and survival rates, additional programs (oral rehabilitation, regular follow-ups, lifestyle changes and cessation of drinking and/or smoking) should be arranged and more professionals, including dentists, consultants, psychologists and speech therapists, should be part of the long-term health care team (Ord et al., 2001; Spencer et al., 2002; Bsoul et al., 2005).
1.2. ORAL PREMALIGNANT LESIONS (OPLs)

Precursors, precancer, premalignant lesions and precancerous lesions are common terms that are used to describe the stages between normal tissue and cancer. In 1997, the World Health Organization (WHO) defined a precancerous lesion as “a morphological altered tissue in which cancer is more likely to occur than in its apparently normal counterpart” (Axell et al., 1996; Pindborg et al., 1997). Clinically, most of OPLs appear as leukoplakia and occasionally as erythroplakia.

1.2.1. Leukoplakia and erythroplakia

Leukoplakia accounts for up to 85% of all precancerous lesions with a prevalence of 1.49-2.6% in the general population (Petti, 2003). The WHO has defined leukoplakia as “a white patch or plaque that can not be characterized clinically or pathologically as any other disease” (Kramer et al., 1978). Because the majority of oral premalignant lesions appear as leukoplakia, the term leukoplakia is frequently used interchangeably with oral premalignant lesions. The male to female occurrence ratio of leukoplakia is about 3:1 (Petti, 2003).

Erythroplakia, compared to leukoplakia, is a rare precancerous lesion with a prevalence of 0.02-0.83% in different geographical regions (Metha et al., 1971; Lay et al., 1982). In 1978, the WHO defined it as “bright red, velvety plaques which cannot be characterized clinically or pathologically as being due to any other condition” (Kramer et al., 1978).
Most patients are diagnosed with erythroplakia while in their 60s and 70s (Feller et al., 1991). The ratio of male to female is about 1.04:1, in other words, no significant difference (Hashibe et al., 2000).

Since the majority of oral premalignant lesions do not become cancers, it is critical to identify those OPLs that will later progress to cancer. Currently the risk prediction of OPLs is through assessment of clinical features and pathological features of OPLs.

1.2.2. Clinical risk factors for oral premalignant lesions

A number of clinical factors have been shown to be associated with increased cancer risk of OPLs. Of these, the following 3 factors are the major risk predictors for assessing an elevated cancer risk of primary OPLs: clinical appearance of nonhomogeneous leukoplakia, location at the floor of mouth and ventrolateral tongue (termed high-risk sites) and a large size.

1.2.2.1. Clinical appearance of OPLs

According to clinical appearance, OPLs can be classified into 2 major types: homogeneous leukoplakia and nonhomogeneous leukoplakia. The latter often also includes erythroplakia, although some sources classify OPLs into 3 categories: homogenous leukoplakia, nonhomogeneous leukoplakia and erythroplakia. Homogeneous leukoplakia is the most common form of OPLs. It has a consistent color –
white, predominantly white, or slightly yellow, and has a consistent texture - a smooth, sometimes slightly wrinkled texture. The majority of leukoplakia does not progress into cancer. The other type, referred to as non-homogeneous leukoplakia, has a mixed color (red and white) or mixed texture (areas of verrucous or nodular texture) or both. This latter form accounts for only 10% of all leukoplakia and has a much higher malignant transformation potential than homogeneous leukoplakia (Axell et al., 1996). There is some evidence that leukoplakia progresses clinically in stages and that some homogeneous leukoplakia develop into a nonhomogeneous leukoplakia over time (Bouquot et al., 1994).

1.2.2.2. Clinical location of OPLs

In Western countries, the majority of oral SCCs develop at the high-risk sites (floor of mouth and ventrolateral tongue). OPLs located at these locations have a much higher risk to become a cancer than OPLs located at other areas of the oral cavity. Studies from our laboratory have shown that OPLs at the high-risk sites has higher frequencies of genetic changes critical for cancer progression than similar OPLs located at low-risk sites, indicating that the location of the OPLs is an independent risk predictor (Zhang et al., 2001).
1.2.3. **Size of OPLs**

Most OPLs are small, and many of these decrease in size or completely regress clinically and do not become cancerous. It is possible that many of these small lesions are reactive lesions and have little malignant potential. However, larger sized lesions have a significantly higher frequency of malignant transformation. Generally, the bigger the premalignant lesion, the more likely it is to transform into cancer. The size of OPLs does not refer to just one lesion but rather the total sum of the sizes of all OPLs in the oral cavity of a patient. The WHO has used size as one of the clinical risk predictors in the staging of OPLs. However, the “cutoff” for the size associated with an elevated risk is not known.

1.2.3. **Histological risk factors for OPLs**

The clinical risk factors help clinicians determine whether a lesion needs to be biopsied. Currently the gold standard for cancer risk of OPLs is histology through the assessment of the presence and degree of dysplasia. In 1997, the WHO defined the term “epithelial dysplasia” as “a pre-cancerous lesion of stratified squamous epithelium characterized by cellular atypia and loss of normal maturation and stratification short of carcinoma in situ”. The existence of epithelial dysplasia in oral lesions predicts a 6.6-36% chance of malignant transformation in the future (Silverman *et al.*, 1984; Pindborg *et al.*, 1997).
The criteria used to diagnose epithelial dysplasia include:

(1) prominent nuclei
(2) altered nuclear shape
(3) higher nuclear/cytoplasmic ratio
(4) increased and abnormal mitotic figures
(5) nuclear and cellular pleomorphism
(6) nuclear hyperchromatism
(7) loss of polarity of cells
(8) abnormal maturation (Pindborg et al., 1997; Coulthard et al., 2003)

Based on the involvement of the epithelial layers by the above features, epithelial dysplasia is further graded as mild (dysplasia involves basal and parabasal epithelial cell layers), moderate (dysplasia involves the lower half of the epithelial layers), severe (dysplasia involves the lower 2/3 of the epithelial layers) and carcinoma in situ (dysplasia involves the full thickness of the epithelial layers but the basement membrane remains intact). The malignant potential of OPLs is directly correlated with the degree of dysplasia. The higher the degree of dysplasia an OPL has, the higher the cancer risk the lesion has (WHO, 1978; Bouquot, 1997; Wright, 1999).
1.3. PROBLEMS IN EARLY DIAGNOSIS

The mortality rate of oral cancer has not improved for the past three decades despite advances in chemotherapy and surgical interventions (Walker et al., 2003). It has one of the worst prognoses among the major human cancers both in terms of mortality and morbidity. This is due largely to the late diagnosis of a significant proportion of the disease. The majority of oral SCCs in developing countries such as India are diagnosed at late stages (stages III or IV), and close to half of oral SCCs in the developed countries are still diagnosed at the late stages. For example, in BC, around 40% of oral SCCs are diagnosed at late stages (III or IV). The 5-year survival rates decline sharply with advancing stages of the cancer: those oral SCCs diagnosed at late stages have only around 20 to 30% of 5-year survival rate, whereas those diagnosed at early stages have a 5-year survival rate around 80% (Walker et al., 2003). Furthermore, late diagnosis results in severe morbidity, more brutal functional compromises and cosmetic issues. This also imposes a greater financial burden on our medical system.

Why are we not diagnosing the disease early? This late diagnosis is particularly surprising considering the fact that we know the main etiological factors (should be able to identify high-risk populations), the disease takes a long time to develop (providing plenty of time to intervene), the oral cavity is visible and we know the clinical presentation of oral premalignant lesions (should be able to identify them), and we have the gold standard histology to guide our risk assessment. Unfortunately current
clinicopathological assessment of risk has multiple problems that cause the late diagnosis of OSCC.

1.3.1. Problems in clinical risk predictors

Clinicians depend upon clinical risk factors to judge whether a lesion is high-risk (cancer or precancer), and if the lesion is judged as high-risk, a biopsy is taken. However, the diagnosis of leukoplakia (white patch) and erythroplakia (red patch) are non-specific because leukoplakia can be very hard to differentiate from reactive non-premalignant hyperplastic lesions (also presenting clinically as white lesions), and erythroplakia can be indistinguishable from inflammatory lesions (also presenting clinically as red lesions). Furthermore, the judgement of whether an OPL is homogeneous or nonhomogeneous frequently requires clinical experience and expertise. Some OPLs are not even clinically apparent (Poh et al., 2007). When small lesions are biopsied, the real lesion sites might be missed due to lack of significant structural changes which can result in false negative reports. Most large OPLs are a mixture of varied grades of dysplasia, hyperplasia and normal tissues (Lumerman et al., 1995). When biopsying large lesions, the most severe dysplastic area might be missed and result in underestimation of the true severity of the lesion. All of these problems frequently prevent a lesion from being biopsied in time or, when a biopsy is taken, the biopsy site does not necessarily represent the area with the greatest dysplastic changes.
I.3.2. Problems in histology (presence and degree of dysplasia) as a risk predictor

Even when a biopsy is taken, the histological hallmarks of OPLs are not universally agreed upon.

I.3.2.1. Pathologist agreement in diagnosing and grading epithelial dysplasia

One problem with using dysplasia as a predictor lies in the subjectivity of the histopathological diagnosis and the requirement of extensive experience in diagnosing borderline lesions. Lack of agreement between oral pathologists in grading and diagnosing dysplasia is a frequent event. Interobserver agreement ranges from 20-50% while intraobserver agreement varies from 50-67% (Abbey et al., 1995; van der Meij et al., 1999). The difficulty lies particularly in the low-grade or borderline lesions because the changes are subtle, and also because reactive lesions can show histological changes resembling low-grade dysplasia. Each oral pathologist interprets and weighs each criterion differently; hence, a lesion may be diagnosed as mild dysplasia by one oral pathologist but diagnosed as normal by another oral pathologist.
1.3.2.2. **Poor correlation between low-grade epithelial dysplasia and oral cancer development**

As mentioned previously, the presence and degree of epithelial dysplasia is used to evaluate the risk of OPLs becoming malignant. This standard is good for judging the malignant risk of high-grade lesions (severe dysplasia and CIS) and guiding the management of these lesions since they are likely to progress into cancer if left untreated. However, epithelial dysplasia is not a good predictor of cancer risk for OPLs with low-grade dysplasia or without dysplasia. The majority of these lesions do not progress into cancers and histology cannot differentiate progressors from non-progressors (WHO, 1978; Bouquot, 1997; Wright, 1999).

1.3.3. **Understanding mechanisms of oral carcinogenesis is the key to find risk predictors**

The development of tools or markers that can differentiate low-grade lesions with high cancer potential from those with low cancer potential would be a breakthrough in cancer research. Such tools/markers will dramatically improve the early detection of high-risk OPLs and prevent oral cancer development by allowing clinicians to identify those lesions requiring aggressive treatment at an early stage. Understanding the mechanism(s) of oral carcinogenesis will facilitate the identification of possible risk predictors.
I.4. GENOMIC AND CHROMOSOME INSTABILITIES

I.4.1. Genomic instability

In 1914, the German biologist Theodor Boveri first hypothesized that multipolar mitosis and aberrant chromosome numbers may lead to cancer development (Boveri, 1914). It is now well known that carcinogenesis, the process of cancer formation, results from accumulation of abnormal genetic events. Many types of genetic changes in cancer cells have been reported including DNA alterations (deletion, insertion, point mutation, and translocation) and chromosome changes (both numerical and structural). The term, **genomic instability**, refers to abnormally high rates (possibly accelerating rates) of genetic change occurring serially and spontaneously in cell-populations, as they descend from the same ancestral cell. By contrast, normal cells maintain genomic stability by operation of elaborate systems which ensure accurate duplication and distribution of DNA to progeny-cells, and prevent duplication of genetically abnormal cells. Many cancer biologists now believe that the loss of stability of the genome is one of the most important aspects of carcinogenesis (Morgan et al., 1996) and that genomic instability not only initiates carcinogenesis, but also allows the tumor cell to become metastatic and evade drug toxicity (Tlsty et al., 1993).

Although the mechanism is still unclear, there are suggestions that genomic instability has three main pathways: nucleotide-excision repair instability (NIN), microsatellite instability (MIN) and chromosomal instability (CIN) (Reshmi et al., 2005). Of these,
CIN draws the most attention for the following two reasons: First, CIN causes the more frequent and more extensive gains and losses of DNA content compared with the other two genetic changes; and second, numerical chromosomal changes are the most prevalent genetic events in the more than 20,000 different solid tumors recorded so far. CIN and MIN are the primary defects in rare or hereditary cancers containing mutations in DNA mismatch repair genes (Mitelman, 1994; Heim et al., 1995).

1.4.2. **Chromosomal instability**

An excess number of chromosomes in tumor cells was first reported over one hundred years ago (Hansemann, 1890; Boveri, 1914). Today, chromosomal aberrations have been reported in almost all malignant tumors (Mitelman et al., 2007). The term, *chromosomal instability*, is used to describe the continuous and significant aberrations to chromosomes, at a rate higher than in normal cells, and is believed to be an important driving force in carcinogenesis (Weaver et al., 2006).

The mechanism of chromosomal instability remains unclear although a few possible pathways have been suggested, mainly telomere dysfunction and a defective mitotic process (Dey, 2004; Rajagopalan et al., 2004; Reshmi et al., 2004).
1.4.2.1. **Telomere dysfunction leads to chromosome instability**

The telomere is composed of 4-15 kbp of DNA sequence (highly repeated hexanucleotides of TTAGGG) capped with associated proteins. The telomeres are located at both terminal ends of each eukaryotic chromosome. The normal functions of telomeres include:

(1) preventing rapid loss of critical genes during DNA replication

(2) controlling the cell life span

(3) capping the ends of chromosomes to prevent chromosome breakage and fusion during DNA segregation

In human and many other organisms, telomeres could be extended by telomerases which is a reverse transcriptase (Joeng et al., 2004; Juola et al., 2006). However, telomerases only exist in stem cells and certain white blood cells but not in other human cells. In human stem cells, telomerases maintain the telomere lengths, which results in the immortality of the stem cells. In other human cells lacking telomerases, there is an average loss of about 65 bp of telomere during each round of DNA replication because the DNA polymerase complex is incapable of replicating the very 5’ ends of both DNA strands. Since the ends of chromosomes are capped with telomere, they are sacrificed during duplication instead of the critical genes at the end of the chromosome. The shortened chromosomes could generate unstable DNA ends leading to more frequent chromosome breakage and fusion during DNA segregation. When the telomere is sufficiently short, the cell enters senescence and p53-dependent apoptosis (Kim et al., 1994; Shay et al., 1997).
Different telomere abnormalities including overexpressed telomerase activity, loss of
capping function and telomere shortening have been reported in different human cancers
(Counter et al., 1992). Some studies showed that overexpressed telomerase maintains
telomere stability, helps cells to become immortal, and facilitates tumor growth indirectly
(Hackett et al., 2002). Other studies reported that when the telomere loses capping
function or shortens, chromosomes break and fuse randomly and this frequently
contributes to CIN (Jin et al., 2002).

1.4.2.2. Defective mitotic processes lead to chromosomal instability

Mitosis is a key step in ensuring precise chromosomal segregation and the separation of
duplicated DNA to daughter cells. Defective mitosis can lead to huge losses or gains of
genes that could result in malignant transformation of human cells. The defect involves
mitotic checkpoints, kinetochores or centrosomes. There are few studies on the roles of
defective mitotic checkpoints and kinetochores in chromosomal instability. In contrast,
defects in centrosomes have been investigated widely because the number of centrosomes
determines the number of mitotic spindles. Supernumerary centrosomes and resulting
multipolar mitosis are frequently found in tissues with chromosomal instability (Sato et
al., 2001; Pihan et al., 2003; Gisselsson et al., 2004). This thesis investigated the
relationship between centrosome abnormalities and oral cancer development. I will
review the centrosome and the potential role of its dysregulation in cancer development
in the following sections.
1.5. CENTROSOME

Centrosomes were first seen and described in purple sea urchin cells (Paracentrotus lividus) the late 19th century by Flemming and Van Beneden (Wilson, 1900). Since then, centrosomes have been found in animal and plant cells and studied widely. In mammalian cells, centrosomes are non-membranous organelles located in the perinuclear cytoplasm.

1.5.1. Centrosome structure

Human cell centrosomes consist of a pair of cylindrical centrioles surrounded by pericentriolar materials. The pair of centrioles is connected perpendicularly to each other with fibrous structures (Figure 1). The diameters and lengths of centrioles are about 100-150nm and 400nm, respectively (Marshall et al., 1999). Centrioles consist of polypeptides including α/β/γ/δ/ε tubulin (Fuller et al., 1995; Dutcher, 2001), centrin (Salisbury, 1995), tektin filaments (Steffen et al., 1994) and other structural proteins (Hinchcliffe et al., 1998). The core of a centriole is a barrel-like structure composed of nine triplet microtubules made of numerous α/β tubulin dimers. Each pair of centrioles includes a mother/mature centriole and a daughter/immature centriole. Mother and daughter centrioles possess minor structural differences. Mother/mature centrioles have appendages including CEP110/centriolin (Ou et al., 2002; Gromley et al., 2003), ninein (Khodjakov et al., 1999; Ou et al., 2000), ε-tubulin (Chang et al., 2003) and possible cenexin (Lange et al., 1995) at their distal ends, γ tubulin at their proximal ends (Fuller et
and several distinct proteins on their outer surfaces, while daughter/immature centrioles lack appendages (Figure 1).

The pericentriolar matrix is composed of a mixture of fibrous, amorphous, and ring-like structures. It has a centromatrix backbone with numerous attached coiled-coil proteins. The centromatrix is a complex lattice composed of 12-15nm thick fibers. It functions as a scaffold and anchors other large coiled-coil proteins (200-450 kDa) associated with microtubule nucleation (Schnackenberg et al., 1999; Ohta et al., 2002), mitotic spindles (Bouckson-Castaing et al., 1996) and cellular regulation pathways (Palazzo et al., 2000). To date, more than 100 regulatory proteins have been found that can anchor to the pericentriolar matrix and this number continues to increase. Among those coiled-coil proteins anchored to the centromatrix, the γ-tubulin ring complexes (γTuRCs) have drawn the most attention and have been studied intensively. In eukaryotes, the γTuRC is an essential component of the microtubule organizing centers and functions as a microtubule nucleator (Job et al., 2003). In mammalian cells, there are two types of γ-tubulin complexes including γTuRCs found in centrosomes and γ-tubulin small complexes (γTuSCs) distributed homogeneously in the cytoplasm. Each γTuSC has two molecules of γ-tubulin and a single molecule of gamma-tubulin complex protein-2 (GCP-2) and GCP-3 while each γTuRC has multiple copies of γTuSCs with GCP-4, GCP-5 and GCP-6. When centrosomes mature during mitosis, γTuSCs are recruited from the cytoplasmic pool to the centrosomes to form γTuRCs and microtubule organizing centers for microtubule nucleation (Murphy et al., 1998; Fava et al., 1999; Murphy et al., 2001). The other important pericentriolar matrix proteins include pericentrin (Doxsey et al.,
1994), centrin, (Baron et al., 1988) centrosomin (Li et al., 1996) and ninein (Bouckson-Castaing et al., 1996).
Figure 1: Structures of the centrosome

(A) α/β tubulin dimers. (B) Numerous α/β tubulin dimers assembled into a microtubule. (C) Three microtubules assembled into a triplet microtubule. (D) Nine triplet microtubules assembled as the core of a daughter/immature centriole. (E) A mother-daughter set of centrioles have a mother/mature centriole and a daughter/immature centriole. The mother centriole has appendages (orange balls) at the distal end while the daughter centriole has none. The centrioles position perpendicular to each other. (F) Each centrosome has two major parts including pericentriolar matrix and a set of mother-daughter centrioles.
1.5.2. **Centrosome cycle**

During the G0 phase, each human cell has one centrosome. During mitosis, both the centrosome and the DNA should duplicate once and divide equally into two daughter cells. The precise duplication and separation of the centrosomes and DNA ensures that both daughter cells will have one centrosome and one correct set of DNA (2N). The process of doubling centrosomes is referred to as the centrosome cycle. Generally, centrosome duplication is a semiconservative process and is cell-cycle dependent. The process can be divided into the 4 steps: centriole splitting, centriole duplication, centrosome maturation and centrosome separation (Andersen, 1999; Hinchcliffe et al., 2001b; Meraldi et al., 2002b) (Figure 2).

1.5.2.1. **Centriole splitting**

This is the first step of centrosome duplication and also is called centriole disengagement or disorientation. During this step, the paired centrioles lose the normal orthogonal relationship, meaning the centrioles are no longer perpendicular to each other. The centrioles separate slightly from each other. In some extreme situations, the centrioles may separate by up to 10μm. The timing of this step is variable and dependent on cell types and their environment. The step can occur at the late G1 phase, telophase or early S phase (Kuriyama et al., 1981; Piel et al., 2000).
1.5.2.2. **Centriole duplication**

Centriole duplication is a complicated process and the mechanism is still unclear. During the S phase, the centrioles duplicate concurrently with DNA duplication. It has been reported that Cdk2/cyclin E or A could play a key role in centriole duplication (Hinchcliffe et al., 1999; Lacey et al., 1999; Meraldi et al., 1999). The centriole duplication starts with a centrin-containing bud growing into a procentriole (daughter centriole) near the proximal surface of the existing centriole during the late G1 phase or early S phase (Rattner et al., 1973). Both newly formed daughter centrioles are slightly separated from the original centrioles and are perpendicular to the original centrioles. The daughter centrioles elongate during the S and G2 phases and reach full lengths at the G2 or M phase.

1.5.2.3. **Centrosome maturation**

Centrosome maturation starts during the late G2 or early M phase. During maturation, additional pericentriolar matrix proteins including more than 3 times the original amount of γ-tubulin are recruited from the cytoplasmic pool to the centrosomes (Khodjakov et al., 1999). During the accumulation of γ-tubulin, microtubule nucleation activity of the centrosomes increases dramatically. At the same time, some specific proteins including ninein and cenexin/Odf2 (Lange et al., 1995; Nakagawa et al., 2001) are also bound to the original daughter centrioles as the daughter centrioles mature to become mother centrioles (Piel et al., 2000).
I.5.2.4. **Centrosome separation**

To form the bipolar mitotic spindles, the two centrosomes need to separate from each other and move to the opposite sides of the nucleus in the mitotic cell. Two events are involved in the process of centrosome separation. First, the two centrosomes or two pairs of mother-daughter centrioles separate from each other. A study conducted in 2001 suggested that centrin phosphorylation might initiate the centrosome separation (Lutz et al., 2001). Second, the two centrosomes move in opposite directions until they are on opposite sides of the cell nucleus. The mitotic spindle then forms between the two centrosomes. Upon division, each daughter cell receives one centrosome. Aberrant numbers of centrosomes in a cell have been associated with abnormal multipolar mitosis.
The centrosome cycle is a semiconservative and cell-cycle dependent process. The cycle has 4 major steps including splitting, duplication/elongation, maturation and separation. (A) Centriole splitting is the first step. The paired centrioles lose their orthogonal relationship and separate slightly from each other during the late G1 phase, telophase or early S phase. (B) Centriole duplication/elongation starts with a centrin-containing bud sitting right on the existing centrioles at late G1 or early S phase. The buds grow into a procentriole and reach the full length at M or G2 phase. (C) The centrosome maturation starts around the late G2 or M phase. During this step, more centrosomal proteins, especially γ-tubulin ring complexes (γTuRCs) are recruited to the pericentriolar matrix and form the microtubule organizing centers. At the same time, ninein and cenexin/Odf2 are also bound to the distal ends of mother centrioles. (D) During centrosome separation, duplicated centrosomes move to opposite sides of the nucleus and form the bipolar mitotic spindle poles to segregate chromosomes.
1.5.3. **Centrosome functions and consequence of dysfunctions**

Centrosomes regulate the cell cycle including the G1/S transition, the G2/M transition, bipolar spindle formation, and exit of cytokinesis, to ensure fidelity of cell division (Figure 3) (Piel et al., 2001; Doxsey et al., 2005). In addition, centrosomes may also play a role in cell shape (Olins et al., 2005), polarity (Lingle et al., 1998), motility (Etienne-Manneville, 2004; Kodama et al., 2004), vesicle trafficking (Hamm-Alvarez et al., 1998; Potokar et al., 2007) and cilia formation. Recent studies have shown that over 100 cellular regulatory proteins could bind to centrosomes and participate in above functions.

In the following sections, I will discuss centrosome functions and cell behavior changes due to centrosome dysfunctions. The normal functions of centrosomes are summarized in Figure 3. And the consequences resulting from abnormal centrosomes are summarized in Figure 4.

### 1.5.3.1. G1/S transition

Emerging evidence supports the role of centrosomes in controlling the G1/S transition. Although the exact mechanism is still unclear, studies have shown that various centrosome proteins are responsible for the G1/S transition. A recent study suggests that 14 centrosome proteins may play a role in the G1/S transition. These proteins include centrosome components (γ-tubulin, pericentrin, GCP-2, GCP-3 and GCP-5), centriole components (δ-tubulin, ε-tubulin and centrin) and regulatory proteins (cell division cycle 14A (Cdc14A) and Cdc14B) (Mikule et al., 2007).
**Disrupt the G1/S transition**

Loss of centrosome function in the G1/S transition results in G1 arrest and supernumerary centrosomes. Supernumerary centrosomes has been used to describe cells with extra copies of centrosomes (Nigg, 2002).

G1 arrest with centrosome disruption has been shown by interfering with centrosome protein synthesis or function and by physical removal of centrosomes. These studies include centriolin silencing with small interfering RNA (siRNA) in telomerase-immortalized human retinal pigment epithelial cells (hTERT-RPE-1 cells) (Gromley et al., 2003); mislocation of A-kinase anchoring protein 450 (AKAP450) with ectopic expression of C-terminus of AKAP450 in HeLa cells (Keryer et al., 2003); and microinjection of antibodies against pericentriolar matrix-1 in murine zygotes (Balczon et al., 2002). Depleting any of the 14 centrosome proteins has been observed to cause G1 arrest of human diploid cells (Mikule et al., 2007). When both centrosomes are destroyed or removed by microsurgery or laser ablation in mammalian cells during mitosis, cells complete mitosis and produce two daughter cells without centrosomes. However, both daughter cells arrest at the G1 phase without proceeding to the S phase during the next round of mitosis (Hinchcliffe et al., 2001a; Khodjakov et al., 2001).

There are a number of consequences of cells arresting at the G1 phase. In Chinese hamster ovary cells treated with 2mM hydroxyurea for 40-60 hours, cells arrest at the G1 phase without proceeding to the S phase. Centrosomes are duplicated a few times without DNA synthesis and result in 3-10 centrosomes in each cell (Balczon et al., 1995).
However, depleting centrosomal proteins in hTERT-RPE-1 cells result in G1 arrest accompanied with defective centrosomes (structural and functional) but no supernumerary centrosomes. The centrosome proteins depleted in this study include γ-tubulin, ε-tubulin, δ-tubulin, pericentrin, GCP-2, GCP-3, GCP-5 and pericentriolar matrix-1. The structurally defective centrosomes include centriole loss, centriole separation and/or centriole fragmentation (Mikule et al., 2007).

1.5.3.2. G2/M transition

Cdk1/cyclin B plays a key role in the G2/M transition in mammalian cells. Phosphorylation of Cdk1/cyclin B was first observed on the centrosome during the prophase by Jackman et al. in 2003 (Jackman et al., 2003). The study showed that integration, activation or inhibition of mitosis-related proteins may occur at the centrosomes. Another study discovered that Cdk1/cyclin B is activated by Cdc25B at the centrosome and controls the G2/M transition (Lindqvist et al., 2005). Either Aurora-A (Cazales et al., 2005) or Polo-like kinase-1 (Kaczanowska et al., 2006) initiates M phase by positively regulating Cdk1/cyclin B (Jackman et al., 2003) through phosphorylation of Cdc25B at the centrosomes. However, checkpoint kinase-1 negatively regulates Cdk1/cyclin B through Cdc25B at the centrosomes during the interphase and prevents entry into the M phase prematurely (Kramer et al., 2004; Schmitt et al., 2006).
Disrupt G2/M transition

Loss of centrosome functions in the G2/M transition results in G2 arrest and abnormalities in both centrosome and chromosome number (aneuploidy).

Disruption of Cdc25B (Lindqvist et al., 2005), Aurora-A (Cazales et al., 2005), Polo-like kinase-1 (Kaczanowska et al., 2006), or checkpoint kinase-1 (Kramer et al., 2004; Schmitt et al., 2006) in cells results in failed mitosis initiation and arrest at the G2 phase. When cells arrest at the G2 phase without entering the M phase, cells remain with 4N DNA instead of the normal 2N DNA. These cells could double DNA to 8N or even further to 16N after additional rounds of endoreplication. When centrosome associated checkpoint kinase-1 is overexpressed in U2OS cells, the U2OS cells first arrest at the G2 phase. After eight days of incubation, U2OS cells have varied DNA contents of 4N (45 ± 2.6%), 8N (12 ± 2.6%), or 16N (3.7 ± 1.2%) with multiple centrosomes (Kramer et al., 2004).

I.5.3.3. Bipolar spindle formation

The number of centrosomes determines the number of mitotic spindles during mitosis. During mitosis, centrosomes duplicate and move to opposite sides of the mitotic cell nucleus. Concurrently, γ-tubulin small complexes (γTuSCs, containing γ-tubulin, GCP-2 and GCP-3) are recruited from the cytoplasmic pools to the proximal ends of the centrioles and form the γTuRCs with GCPs 4, 5 and 6. γTuRCs are essential components of microtubule organizing centers, and nucleating microtubules. Microtubules grow
continuously from the centrosomes and become stabilized after attaching to the kinetochores of each duplicated chromosome. Two sets of microtubules grow from the two centrosomes located on opposite sides of the nucleus and form the bipolar spindles. The duplicated chromosomes are aligned at the center of the dividing cell and are segregated symmetrically into two identical daughter cells.

**Multipolar mitosis**

One of the important functions of the centrosome is to form bipolar spindles to segregate chromosomes symmetrically into two daughter cells. With more than two centrosomes, there are two possible consequences. One is supernumerary centrosomes clustering into two groups to form bipolar spindles (Quintyne *et al.*, 2005). The other is supernumerary centrosomes forming multipolar mitosis. In one study, involving more than 200 p53-/- mouse embryonic fibroblasts going through mitosis (i.e., all with complete loss of p53 function), 34% of the mouse embryonic fibroblasts had more than 2 (3-25) centrosomes. Ten percent of the mouse embryonic fibroblasts with supernumerary centrosomes formed multipolar mitotic spindles. In contrast, all of the control mouse embryonic fibroblasts with wild type p53 (p53 +/-) went through normal bipolar mitosis (Sluder *et al.*, 2004).

On average, about 80% of multipolar mitosis is tripolar mitosis (Eckl, 1993). When multipolar spindles are formed, each mitotic spindle pole could have equal or unequal influence on the chromosomes. Unequal influence between mitotic spindles resulted in asymmetrical chromosomal segregation and daughter cells with different amounts of DNA (Saunders *et al.*, 2000). Most daughter cells won’t survive due to the lack of some
essential genes. The surviving daughter cells have randomly varied amounts of DNA contents compared to normal cells (Juul et al., 1991).

1.5.3.4. Cytokinesis

Several lines of study have shown a possible role of centrosomes in cytokinesis. The most convincing evidence comes from the observation of mitosis in mammalian cell cultures through physical destruction of centrosomes or interference of centrosome proteins. These studies are discussed in the following sections.

Cytokinesis failure

Loss of centrosome functions during cytokinesis results in incomplete or defective cytokinesis and abnormalities in both centrosomes and DNA (aneuploidy).

After centrosomes are destroyed by laser ablation in rat kangaroo kidney epithelial cells, 30-50% of the cells fail to complete cytokinesis. These abnormal cells are still connected with a cytoplasmic bridge and could not separate into two daughter cells (Khodjakov et al., 2001). Incomplete or defective cytokinesis has also been reported in the following studies: centriolin silencing by siRNA in hTERT-RPE-1 cells (Gromley et al., 2003); dislocation of endogenous AKAP450 by using ectopic expression of the C-terminus of AKAP450 in HeLa cells (Keryer et al., 2003); Cdc14A silencing by siRNA in U2OS cells (Mailand et al., 2002); Cep55 silencing by siRNA in HeLa (Fabbro et al., 2005).
The most common consequence of cytokinesis failure is ‘binucleate and hyperploid’
daughter cells. The other possible consequences include mononucleate cells,
multinucleate cells (Doxsey, 2001; Uetake et al., 2004), multicellular syncytia, persistent
intercellular bridges and apoptotic cell deaths (Kaiser et al., 2002; Gromley et al., 2003).
Most daughter cells can’t enter subsequent mitosis. Even if these daughter cells enter
subsequent mitosis, the chance of multipolar mitosis increases substantially (Sluder et al.,
2004). Overexpression of Aurora-A in HeLa cells results in multinucleate cells
especially binucleate cells. 40% of multinucleate cells have 4N DNA, 25% have 5-6N
DNA and 30% have 7-8N DNA (Meraldi et al., 2002a). In HeLa cells, dislocation of
AKAP450 induces cytokinesis failure with a higher frequency of tetraploid cells (28.5 ±
3.3%) compared to control cells (10 ± 3.5%). Among the tetraploid cells, 39-50% are
binucleate. Only 1.7-3% of the binucleate cells with 4N DNA go through subsequent
mitosis (Keryer et al., 2003).

1.5.3.5. **Cell shape, polarity, motility, vesicle trafficking and cilia formation**

Centrosomes play indirect and possibly direct roles in controlling cell shape, motility and
vesicle trafficking through microtubules. In human cells, microtubules are organized into
an astral (centrosomal) or a non-astral (non-centrosomal) microtubule array. The astral
microtubule arrays only exist in proliferating cells, migrating cells like neutrophils and
cell cultures. Most polarized and nonmigratory cells like epithelial cells only have few
microtubules attached to their centrosomes; most microtubules do not attach to their
centrosomes but organize into linear non-astral microtubule (non-centrosomal) arrays
(Bartolini et al., 2006). Microtubules organize into different structural cytoskeletons depending on the cell type and thus control the cell shape and polarity (Rodionov et al., 1993). Microtubules also work with actins and intermediate filaments in controlling cell motility (Etienne-Manneville, 2004; Kodama et al., 2004) and vesicle transport (Hamm-Alvarez et al., 1998; Potokar et al., 2007). In cytotoxic T lymphocytes, the delivery of cytotic granules to the immunological synapse is mediated by centrosomes rather than by actins or microtubules (Stinchcombe et al., 2006).

Disruption of cell shape, motility, polarity, cilia formation and vesicle trafficking

Abnormal centrosomes could disorganize microtubule networks and increase microtubule nucleation activity. The disruption of microtubules would influence cell shape, polarity, motility and vesicle trafficking. The overexpression of γ-tubulin (centrosomal protein) in monkey kidney COS cells generate multiple, varied-sized centrosomes scattered in the cytoplasm and reorganize microtubule networks poorly (Shu et al., 1995). Another study shows multiple large centrosomes (average 4.3 centrosomes in each cell), loss of histological differentiation, and dramatically increase microtubule nucleation activity (about 10-fold) in breast cancer cells (Lingle et al., 1998). In human prostate cancer, the levels of centrosome abnormalities is also strongly correlated with Gleason grades (Pihan et al., 2001). Both above studies suggest that centrosome abnormalities influence cell shape and tissue differentiation through abnormal microtubule nucleation.
Figure 3: Summary of normal functions of centrosome and the proteins involved in each function

Studies have reported that centrosomes are associated with many different cell functions. (A) At the G0 phase, centrosomes are involved with cell motility, cell shape, cell polarity, vesicle trafficking and cilia formation. Centrosomes are also related to cell cycle regulation including G1-S transition (B), G2/M transition (C), bipolar spindles formation (D) and the exit of cytokinesis (E). Dysregulated expression or dislocation of centrosomal proteins (shown in blue italic) could disrupt cell cycle progression.
Figure 4: Summary of consequence of centrosome abnormalities
(A) Abnormal centrosomes disorganize microtubules, which disrupt cell motility, cell shape, cell polarity, cilia formation and vesicle trafficking. (B) Abnormal centrosomes disrupt G1-S transition and arrest cells at the G1 phase. Cells could have supernumerary centrosomes after additional rounds of endoreplication of centrosomes. However, some cells have defective centrosomes rather than supernumerary centrosomes. (C) Abnormal centrosomes disrupt G2/M transition and cause cells arrested at the G2 phase. Cells could undergo additional rounds of endoreplication of both DNA and centrosomes. Cells have 4N, 8N or 16N DNA and supernumerary centrosomes. (D) Abnormal centrosomes increase the chance of multipolar mitosis and produce more than 2 daughter cells with varied amounts of DNA content. (E) Abnormal centrosomes could induce cytokinesis failure and primarily produce binucleate cells with 4N DNA and 2 centrosomes. Binucleate cells frequently go through multipolar mitosis during subsequent mitosis and produce more than 2 daughter cells with varied amounts of DNA content.
1.5.4. **Mechanisms underlying centrosome abnormalities**

Many studies have investigated centrosome abnormalities at the molecular/protein level by using mammalian cell cultures. The possible pathways leading to centrosome abnormalities are summarized in Figure 5 and include:

1. centrosome overduplication
2. centrosome accumulation due to cytokinesis failure
3. centrosome splitting
4. formation of acentriolar centrosomes
5. dysregulation of centrosomal proteins

Both centrosome overduplication and centrosome accumulation result in increased number of centrosomes. The last three pathways (centrosome splitting, formation of acentriolar centrosomes and dysregulation of centrosomal proteins) lead to production of abnormal centrosomes. In the following sections, I will review each pathway leading to centrosome abnormalities.
Figure 5: Summary of possible pathways leading to centrosome abnormalities

(A) Centrosome overduplication results from more than one round of centrosome duplication cycle during mitosis. It occurs when cells arrest at the G1 phase and the centrosome duplication cycle dissociates from the DNA replication cycle. (B) Centrosome accumulation is the consequence of cytokinesis failure during mitosis. Both duplicated centrosomes and DNA cannot be segregated successfully and accumulated in the cell. (C) Centrosome splitting means the abnormal separation of the paired centrioles during the G0 phase. Each centrosome contains one centriole instead one pair of centrioles. (D) Acentriolar centrosomes are centrosomes with missing centrioles. (E) Dysregulated centrosomal proteins are altered expression of centrosomal proteins. This change is reflected in the change of centrosome size but not centrosome number.
1.5.4.1. **Centrosome overduplication**

Centrosome overduplication plays a significant role in centrosome abnormalities. During normal mitosis, the DNA duplication cycle couples with the centrosome cycle and both DNA and centrosomes are duplicated once only. A study conducted in 1995 showed that the centrosome cycle could be disassociated from the DNA duplication cycle under certain conditions. When Chinese hamster ovary cells were treated with hydroxyurea or aphidicolin, the centrosome duplication cycle was disassociated from the DNA duplication cycle and mitosis. Cells arrested at the G1 phases without DNA duplication. However, centrosomes continued with additional rounds of duplication cycles causing supernumerary centrosomes in each cell (Balczon *et al.*, 1995). Further studies using mouse embryonic fibroblasts reveal that loss of p53 would lead to supernumerary centrosomes in a single cell cycle (Fukasawa *et al.*, 1996). Similar results have also been observed in the following situations: overexpression of Mdm2 (Carroll *et al.*, 1999), overexpression of Dbf2-related kinase (Hergovich *et al.*, 2007), inactivation of p21 (Duensing *et al.*, 2006), inactivation of S-phase kinase-associated protein 2 (Nakayama *et al.*, 2000), depletion of Tax1 binding protein 2 (Ching *et al.*, 2006), overexpression of cyclin E with loss of p53 (Mussman *et al.*, 2000) and inhibition of BRCA1 (Ko *et al.*, 2006). Another study showed that viral oncoprotein, HPV-16 E7 (Duensing *et al.*, 2002b; Duensing *et al.*, 2002a) and Human T-cell leukemia virus type I Tax protein (Ching *et al.*, 2006) could also cause centrosome overduplication. HPV-16 E7 oncoprotein dysregulates Cdk2 activity and overduplicates centrosomes during the early stage of the disease before noticeable phenotype changes (Duensing *et al.*, 2001). Human T-cell
leukemia virus type I Tax oncoprotein interacted with Tax1 binding protein 2 (centrosomal protein) and induced amplification of centrosomes (Ching et al., 2006).

1.5.4.2. **Centrosome accumulation due to cytokinesis failure**

Several studies show that cytokinesis/cleavage failure could result in centrosome accumulation. When p53-incompetent rat embryo fibroblasts and wild type rat embryo fibroblasts are treated with hydroxyurea, cytokinesis/cleavage failure is induced and results in centrosome accumulation. The centrosome accumulation is similar in both p53-incompetent rat embryo fibroblasts (about 20%) and wild type rat embryo fibroblasts (about 15%) 60 hours after the hydroxyurea treatment. The study shows that centrosome accumulation is independent of p53 status (Borel et al., 2002). Another study conducted in 2002 reveals that Aurora-A overexpression in HeLa cells also result in cytokinesis failure (tetraploidization and multinucleation) and centrosome accumulation. The study supports the belief that Aurora-A caused centrosome accumulation through cytokinesis failure rather than through overduplication. Although centrosome accumulation is independent of p53 status, absence of p53 exacerbated centrosome accumulation induced by overexpressed Aurora-A in p53-/- mouse embryonic fibroblasts. When Aurora-A is overexpressed, 25% of wild type mouse embryonic fibroblasts and 80% of p53-/- mouse embryonic fibroblasts show centrosome accumulation (> 2 centrosomes in each cell) (Meraldi et al., 2002a).
1.5.4.3. **Abnormal centrosome splitting or centrosome fragmentation**

Normally, each centrosome contains two centrioles connected by a fibrous matrix. Centrioles split during the centrosome duplication cycle at the mitosis stage. Under some abnormal conditions, the paired centrioles separate and become two “split or half” centrosomes containing only one centriole each during interphase. The actual mechanisms are still unclear. Several studies using mammalian cell culture systems show that centrosome cohesion and integrity are regulated under the balanced activities of kinases and phosphatases. Centrosome splitting/fragmentation could be triggered by depletion of HEF-1 (Human Enhancer of Filamentation 1) (Pugacheva et al., 2005), overexpression of NIMA (never in mitosis gene a)-related kinase 2 (Mi et al., 2007), Ran-binding protein 1 (Di Fiore et al., 2003), Cdk2/cyclin E or Cdk2/ cyclin A (Meraldi et al., 2001). Depletion of protein phosphatase 1 also enhances centrosomal splitting (Mi et al., 2007). Other situations resulting in centrosomal splitting include CGL-2 cells treated with 2μmol/L. arsenite for 20 hours (Yih et al., 2006), U2OS cells treated with 5μg/ml nocodazole for one hour (Meraldi et al., 2001), and existence of damaged DNA in Chinese hamster ovary cells (Hut et al., 2003).

1.5.4.4. **Formation of acentriolar centrosomes or centrosome**

Little is known about acentriolar centrosomes and mechanisms by which they are produced. Amplified acentriolar centrosomes (multiple centrosomes with missing centrioles) have been reported in CGL-2 cells, a hybrid (ESH5) of the HeLa variant,
treated with 2μmol/L arsenite for 20 hours (Yih et al., 2006). Similar findings have also been reported in human breast BT-549 cell lines (11.2%, n = 223) and Hodgkin’s disease derived cell lines (14.1%, n = 227) (Pihan et al., 1998). A study has shown the formation of multiple acentriolar centrosomes (containing γ-tubulin and pericentrin) in the cytoplasm of vertebrate cells 5-8 hours after centrosomes were artificially destroyed by laser microsurgery (Khodjakov et al., 2002). This illustrates the ability of cells to assemble acentriolar centrosomes through de novo pathways which are normally suppressed. There should be a mechanism controlling the de novo centrosome assembly and dysregulation of this mechanism could lead to amplified acentriolar centrosomes.

1.5.4.5. **Dysregulation of centrosomal proteins**

Centrosomal proteins include four groups of proteins:

1. proteins that form the structures of centrosomes
2. proteins that function in microtubule formation
3. proteins that link both microtubule nucleator proteins and regulatory proteins
4. regulatory proteins such as kinases, phosphatases and signalling proteins

(Lange, 2002)
More than 100 regulatory proteins bind to centrosomes at different stages of the cell cycle. Recent studies show that most regulatory proteins are activated after being bound to the centrosome and responsible for different functions including cell cycle progression, apoptosis, DNA damage response, cytoplasmic trafficking, etc (Doxsey et al., 2005).

Altered expression or dislocation of centrosomal proteins could disrupt the G1/S transition, the G2/M transition and the completion of cytokinesis. In cell cultures, altered expression of centriolin (Gromley et al., 2003), pericentriolar matrix-1 (Balczon et al., 2002), γ-tubulin, δ-tubulin, ε-tubulin, pericentrin, GCP-2, GCP-3, GCP-5 (Mikule et al., 2007), Cdc14A (Mailand et al., 2002), Cdc14B, Cdk1/cyclin B (Jackman et al., 2003), Cdc25B (Lindqvist et al., 2005), Aurora-A (Cazales et al., 2005), Polo-like kinase-1 (Kaczanowska et al., 2006) or Cep55 (Fabbro et al., 2005) disrupt cell cycle progression and dramatically increase the chances of multipolar mitosis and polyploidy cells. Overexpression of Aurora-A (Tong et al., 2004), Plk-1 (Yamamoto et al., 2006) or cyclin E (Koutsami et al., 2006) also causes similar consequences in human cancers.
1.6. CENTROSOME ABNORMALITIES AND CIN IN CANCER DEVELOPMENT

The loss of genomic stability is believed to be one of the most important aspects of carcinogenesis (Morgan et al., 1996). Genomic instability not only initiates carcinogenesis but also facilitates cancer progression and evasion of drug toxicity (Tlsty et al., 1993). CIN may be the most important pathway of genomic instability since chromosome aberrations have been found in most malignant tumors. CIN has been widely studied by using various techniques including traditional metaphase cytogenetics (karyotypes) or interphase cytogenetics (FISH, fluorescence in situ hybridization) and array comparative genomic hybridization (array CGH) (Dey, 2004).

I.6.1. CIN in oral cancer and OPL

I.6.1.1. CIN in oral cancer

A variety of techniques have been used to demonstrate the presence of CIN in oral cancer. Using traditional metaphase cytogenetics, a recent study analyzed the karyotypes of 106 short-term cultured OSCCs. The results showed karyotypic changes in all cultures with 64% of tumors displaying highly complex karyotypes and the remaining 36% displaying simple karyotypic changes (Jin et al., 2006). The common karyotypes in OSCC are near-triploid chromosome numbers (Reshmi et al., 2004; Jin et al., 2006). On average, cancer cells at late stages have 60-90 chromosomes in each cell. Although varied chromosome numbers have been reported within and between clones of the same OSCC cell line, gains
or losses of some specific chromosomes were also noticed (Jin et al., 2002). Among these 106 short-term cultures of OSCCs, the most common numerical changes of chromosomes are the losses of chromosomes Y, 21, 22, 18, 19, and 20 and gains of chromosomes 7, 9, 16, 8, and 18. The most common structural changes of chromosomes are the unbalanced rearrangement around the centromeric regions of chromosome 3, 8, 11, 5, 13, 14 and 15 (Jin et al., 2006). It is important to note that the different cell culture media may selectively affect the growth of certain cell populations in the tumor and thus influence the results of karyotype analysis (Jin et al., 1993).

A number of studies have used fluorescence in situ hybridization (FISH), including multicolor FISH, to determine copy number changes of specific genes or number changes of chromosomes in OSCC in cell cultures or tissue sections. The study has shown losses of chromosomal regions containing cancer-associated genes on 1p and 8p and amplification of Hsr and CCND1 at 11q13 (Jin et al., 2006). Studies from our laboratory have also shown gains of epidermal growth factor receptor (EGFR) and Cyclin D1 genes in oral cancers as well as an elevation in the frequency of cells with trisomy 7 and 11 in oral dysplasia (unpublished data).
Studies using microsatellite analysis for loss of heterozygosity (LOH) in archival oral tissue sections also indirectly supports the role of CIN in oral carcinogenesis. LOH is caused by a variety of mechanisms including chromosome nondisjunction, gene conversion or recombination or gene amplification. Many chromosomal regions showing frequent LOH contain tumor suppressor genes such as \textit{FHIT}, \textit{P16}, \textit{TP53} and \textit{cyclin D1} (Rosin \textit{et al.}, 2000; Zhang \textit{et al.}, 2001; Rosin \textit{et al.}, 2002).

More recent studies have also used array CGH to detect DNA copy number alterations in OSCC. DNA copy number alterations include the entire arm of chromosomes, segmental DNA copy number changes and gene size alterations. Our research team has reported abnormal amplification at 7p11 (containing \textit{EGFR}), 11q13 (containing \textit{cyclin D1}) (Garnis \textit{et al.}, 2004a), 3p21 (Garnis \textit{et al.}, 2003), 11q22.28-22.3 (Baldwin \textit{et al.}, 2005) and 8q21-24 (Garnis \textit{et al.}, 2004b) in OSCCs using array CGH.

1.6.1.2. \textbf{CIN in OPLs}

Studies using DNA cytometry, FISH, array CGH and LOH have reported the existence of CIN in OPLs. A hamster cheek-pouch carcinogenesis model study using DNA cytometry (computer imaging technology) has shown an increased frequency of aneuploidy in oral premalignant lesions. The mean DNA index is 2.22 in control cells, 2.93 in OPLs, 3.56 in carcinoma \textit{in situ} and 4.35 in OSCC (Raimondi \textit{et al.}, 2005). Another study using array CGH has reported DNA copy number changes (gains on 8q, 16p and losses on 3p, 13q ,4q, 8p and 9p) in 12 human OPLs. This suggests that aneuploidy is a very early
event in oral carcinogenesis (Weber et al., 1998). Genomic profiling of archival tissues led to the discovery of frequent amplifications of 8q21-24 in OPLs (Garnis et al., 2004a). In our laboratory, we have observed more frequent incidences of trisomy 7 and 11 in OPLs using FISH (unpublished data). LOH was frequently found in multiple chromosome arms in OPLs without dysplasia or with minimal dysplasia (Rosin et al., 2002). We have also found that increased numbers of chromosome arms with LOH (an indication of genomic instability) in OPLs is associated with higher risk of cancer formation (Rosin et al., 2000). The above findings strongly support a significant role of CIN in oral carcinogenesis.

1.6.2. **Relationship between centrosome abnormalities and CIN in cancer development**

1.6.2.1. **Centrosome abnormalities are closely associated with CIN during cancer development**

The centrosome is an important cell cycle regulator involved in the G1/S transition, the G2/M transition, bipolar spindle formation and exit of cytokinesis. Abnormal centrosomes would lead to cell cycle dysregulation, cells with polyploid DNA, and multipolar mitosis. It is therefore hypothesized that centrosomes likely play a critical role in CIN and cancer development. Numerous studies have supported this theory by demonstrating a close relationship between CIN, multipolar mitosis and centrosome
abnormalities in various tumor types using animal models, human cancer cell lines, and human cancers.

In 2001, Shono et al. orthotopically transplanted human pancreatic carcinoma cells into nude mice and observed a positive correlation between numerical chromosomal alterations, multipolar mitosis and multiple centrosomes. The data has shown a step-wise progression of centrosome abnormalities in local (5.7-fold) and metastatic tumors (7.3-fold in liver and 8.4 fold in peritoneal cavity) (Shono et al., 2001). A similar correlation between numerical chromosomal alterations, multipolar mitosis and centrosome abnormalities has also been reported in 8 human pancreatic cell lines (AxPC-1, BxPC-3, H-48 N, KP-1N, KP-2, KP-3, Panc-1 and SUIT-2). This study used FISH with DNA probes to chromosomes 1 and 8 to ascertain CIN frequencies. A positive correlation was reported between centrosome abnormalities and instability of both chromosomes (Sato et al., 2001). In human cancer tissues, a significant correlation ($P < 0.005$) between centrosome abnormalities and gain of chromosome 8 has also been reported in carcinoma in situ of human prostate, cervix and breast using FISH analysis on archival tissue sections (Pihan et al., 2003).

**I.6.2.2. Centrosome abnormalities lead to chromosomal instability**

A close relationship between centrosome abnormalities and CIN in various cancers has been reported by many studies using different samples and techniques. Researchers have questions whether centrosome abnormalities are the origin or the consequence of
chromosomal instability. A majority of studies have suggested that centrosome abnormalities lead to multipolar mitosis and numerical chromosome changes while some other studies have suggested that centrosome abnormalities are the consequence of genomic instability. Current research evidence tends to support the hypothesis that centrosome abnormalities lead to chromosomal instability.

One study supporting the hypothesis that centrosome abnormalities lead to chromosomal instability found that centrosome abnormalities precede CIN in the precancerous lesions. Pihan et al. (2003) studied centrosome abnormalities and CIN in carcinoma in situ (CIS) samples from human uterine cervix, prostate and female breast. The study found that there were more cells with centrosome abnormalities and lacking CIN than cells with CIN and lacking centrosome abnormalities. This finding suggests that centrosome abnormalities occur prior to CIN.

Other studies have shown that direct manipulation of centrosome protein expression in cell culture models leads to numerical chromosome changes and malignant transformation of cells. In 2001, Pihan et al. induced centrosome abnormalities and CIN in vitro. Normal prostate cell lines (1542-NPTX) were transfected with a full length of hemagglutinin-tagged pericentrin. In the pericentrin-expressing 1542-NPTX cells, the frequency of centrosome abnormalities was about 20% compared to 1% in control cells. Disorganized mitotic spindles and abnormal mitosis existed in these pericentrin-expressing cells. Pericentrin-expressing 1542-NPTX cells also contained more than double the DNA content compared to control cells (Pihan et al., 2003). In another study,
overexpression of the centrosome associated checkpoint kinase-1 in U2OS cells reduced cyclin dependent kinase 1 (Cdk1) activity and arrested cells at the G2 phase. After eight days of incubation, U2OS cells had DNA contents of 4N (45 ± 2.6%), 8N (12 ± 2.6%), or 16N (3.7 ± 1.2%) (Kramer et al., 2004). These results support the concept of a potentially causative role of centrosome aberrations in CIN and malignant progression.
I.7. CENTROSOME ABNORMALITIES AT MICROSCOPIC LEVEL IN PARAFFIN EMBEDDED CANCER TISSUES

Centrosome abnormalities in paraffin-embedded cancer tissues could be detected by using fluorescence immunostaining with antibodies to γ-tubulin, pericentrin or centrin. Under the fluorescence microscope, structural and numerical centrosome abnormalities have been classified into categories of size, shape, number, presence/absence of amplification and grades of amplification (Carroll et al., 1999; Sato et al., 1999; Gustafson et al., 2000; Kuo et al., 2000; Pihan et al., 2001; Nakajima et al., 2004; Hsu et al., 2005; Koutsami et al., 2006). In the following sections, I will review the common criteria used in evaluating centrosome abnormalities. The study results are summarized in Table 1 below.

I.7.1. Size abnormality

When reporting centrosome size abnormalities in human cancers, different criteria have been used in different studies. Generally, size abnormalities of centrosomes are defined two ways. The first method is by comparing centrosome sizes (diameter) of cancer cells with those in other types of cells (generally fibroblasts and lymphocytes) from the same tissue section. Abnormal centrosomes in cancer cells have diameters greater than twice that of centrosomes in other types of cells (lymphocytes or fibroblasts) (Sato et al., 1999; Kuo et al., 2000; Jiang et al., 2003; Pihan et al., 2003; Nakajima et al., 2004; Hsu et al., 2005). The second method is by comparing centrosome sizes in cancer cells with the
centrosome sizes of their normal counterparts (e.g., SCC cells with normal squamous epithelial cells). Abnormal centrosomes in cancer cells have diameters greater than twice that of the average diameter of centrosomes in their normal counterparts (Pihan et al., 2003). To better observe the pattern of centrosome size change in my thesis, I used computer software to measure centrosome diameters.

1.7.2. **Shape abnormality**

Some research papers have reported that supernumerary centrosomes may aggregate into specific shapes around the nucleus. These shapes include clusters (patch pattern) and string-like structures (> 3μm). The mechanism and significance of these shape abnormalities is still unclear. These shape abnormalities of centrosomes do not exist in every type of human cancer but have been reported in human malignancies of the prostate (Pihan et al., 1998; Pihan et al., 2001), gall bladder (Kuo et al., 2000) and ovary (Hsu et al., 2005). In my thesis, I evaluated shape change of centrosomes as cluster formations and string-like structures.

1.7.3. **Number abnormality**

Numerical centrosome abnormalities (> 2 centrosomes per cell) are reported in all papers describing centrosome abnormalities (Sato et al., 1999; Kuo et al., 2000; Pihan et al., 2003; Nakajima et al., 2004; Hsu et al., 2005). In my thesis, I further subcategorized number abnormalities of centrosomes into two groups based on the size and location of the centrosomes. The two groups are: > 2 centrosomes in a localized area of the
cytoplasm of the cell; and multiple, irregular sized centrosomes scattered in the cytoplasm of the cell.

**I.7.4. Presence/absence of centrioles in centrosomes**

A normal centrosome should contain both a pericentriolar matrix and centrioles. The term 'acentriolar centrosome' has been used to refer to centrosomes missing centrioles. The absence of centrioles in abnormal centrosomes has been evaluated in cancer cell lines but not in paraffin-embedded human tissues. Centrosomes missing centrioles (acentriolar centrosomes) were first reported by Pihan et al. in 1998. In this study, centrosomes were detected by anti-pericentrin antibody while centrioles were detected by anti-α tubulin antibody. Acentriolar centrosomes were noted in breast cancer cell line BT-549 (11.2%) and Hodgkin's disease derived cell line L428 (14.1%) (Pihan et al., 1998). In my thesis, I examined the patterns of centrioles in amplified centrosomes by using two different antibodies to detect centrosomes and centrioles, respectively.

**I.7.5. Presence/absence and grades of amplification**

The presence/absence of centrosome amplification has been reported by studies in non-small cell lung cancer (Koutsami et al., 2006) and squamous cell carcinoma of the head and neck (Carroll et al., 1999). Another study evaluated the severity of centrosome amplification in oral cavity squamous cell carcinoma by grading its presence using percentage ranges (Gustafson et al., 2000).
Table 1: Summary of centrosome abnormalities in cancers in studies using formalin-fixed paraffin embedded tissues

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<thead>
<tr>
<th>Reference</th>
<th>Tissue</th>
<th>Categories of samples</th>
<th># of samples</th>
<th>Size</th>
<th>Number</th>
<th>Shape</th>
<th>Total (size, number, shape)</th>
<th>Presence/absence or grades of amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koutsami et al. 2006</td>
<td>Lung</td>
<td>Cancer-adjacent normal</td>
<td>68</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyperplasia</td>
<td>13</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0% of cases</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diploid NSCLC</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>10% of cases</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aneuploid NSCLC</td>
<td>33</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>70% of cases</td>
<td>NA</td>
</tr>
<tr>
<td>Hsu et al. 2005</td>
<td>Ovary</td>
<td>Normal</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0% of cases</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade I</td>
<td>23</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>60.9% of cases</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade II</td>
<td>30</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>83.3% of cases</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade III</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>100% of cases</td>
<td>NA</td>
</tr>
<tr>
<td>Nakajima et al. 2004</td>
<td>Liver</td>
<td>Diploid HCC</td>
<td>22</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.54 ± 0.51% of cells/case</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non-diploid HCC</td>
<td>11</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.58 ± 1.59% of cells/case</td>
<td>NA</td>
</tr>
<tr>
<td>Pihan et al. 2001</td>
<td>Prostate</td>
<td>Cancer-adjacent normal</td>
<td>128</td>
<td>100% as baseline</td>
<td>100% as baseline</td>
<td>NA</td>
<td>NA</td>
<td>None: 17% of cases 1+ (few cells):22% 2+ (&gt;10%): 50% 3+ (&gt;20%): 11%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gleason grade II + III</td>
<td>12</td>
<td>~180%</td>
<td>~120%</td>
<td>NA</td>
<td>NA</td>
<td>None: 17% of cases 1+ (few cells):22% 2+ (&gt;10%): 50% 3+ (&gt;20%): 11%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gleason grade: IV + V</td>
<td>12</td>
<td>~260%</td>
<td>~142%</td>
<td>NA</td>
<td>NA</td>
<td>None: 17% of cases 1+ (few cells):22% 2+ (&gt;10%): 50% 3+ (&gt;20%): 11%</td>
</tr>
<tr>
<td>Gustafson et al. 2000 **</td>
<td>Oral Cavity</td>
<td>Cancer-adjacent normal</td>
<td>18</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>None: 6% of cases 1+: 27% of cases 2+: 50% of cases 3+: 17% of cases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Squamous cell carcinoma</td>
<td>18</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>None: 6% of cases 1+: 27% of cases 2+: 50% of cases 3+: 17% of cases</td>
</tr>
<tr>
<td>Reference</td>
<td>Tissue</td>
<td>Categories of samples</td>
<td># of samples</td>
<td>Size</td>
<td>Number</td>
<td>Shape</td>
<td>Total (size, number, shape)</td>
<td>Presence/absence or grades of amplification</td>
</tr>
<tr>
<td>----------------</td>
<td>------------</td>
<td>---------------------------------------</td>
<td>--------------</td>
<td>------</td>
<td>--------</td>
<td>-------</td>
<td>-----------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Kuo et al. 2000</td>
<td>Gallbladder</td>
<td>Benign</td>
<td>37 *</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.05% of cases</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cancer</td>
<td>40 **</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>60% of cases</td>
<td>NA</td>
</tr>
<tr>
<td>Sato et al., 1999</td>
<td>Pancreas</td>
<td>Normal</td>
<td>12</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chronic pancreatitis</td>
<td>5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenoma</td>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>66% of cases</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenocarcinoma</td>
<td>13</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>85% of cases</td>
<td>NA</td>
</tr>
<tr>
<td>Carroll et al., 1999</td>
<td>Head and Neck</td>
<td>Cancer-adjacent normal</td>
<td>12</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0% of cases</td>
<td>83% of cases (n=10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCCHN</td>
<td>12</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Breast</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cancer-adjacent normal</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ductal carcinoma</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>75% of cases</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: SCCHN, squamous cell carcinoma of head and neck; NSCLC, non-small cell lung cancer; HCC: hepatocellular carcinoma.

NA: not available

* Benign biliary lesions include benign gallbladder disease, hepatolithiasis, and choledochal cyst

** Biliary cancers include gallbladder cancer, cholangiocellular carcinoma and bile duct cancer.

** In the Gustafson study, samples were categorized as follows: 0: no cells with centrosome amplification; 1+: few cells; 2+: >10% of cells; 3+: >20% of cells.

Definition of centrosome abnormalities:

1. Size: greater than twice centrosome diameters in non-tumor cells in the same section or normal epithelium adjacent to tumors.
2. Number: > 2 centrosomes per cell
3. Shape: cluster or string-like structure with length > 3 μm.
4. Total: the sum of centrosome abnormalities including size, number and shape if any presented
5. Amplification: multiple, irregular, varied sized centrosomes scattered in the cytoplasm
Table 2: Summary of centrosome abnormalities in precancers in studies using formalin-fixed paraffin embedded tissues

<table>
<thead>
<tr>
<th>Reference</th>
<th>Tissue</th>
<th>Categories of samples</th>
<th># of samples</th>
<th>Size</th>
<th>Number</th>
<th>Shape</th>
<th>Total (size, number, shape)</th>
<th>Amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kayser et al. 2005</td>
<td>Colon</td>
<td>Cancer-adjacent normal</td>
<td>21</td>
<td>1 as baseline</td>
<td>0.878 centrosome/cell</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LGIN</td>
<td>27</td>
<td>2.035</td>
<td>1.787</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HGIN</td>
<td>16</td>
<td>2.573</td>
<td>2.259</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenocarcinoma</td>
<td>33</td>
<td>2.582</td>
<td>2.267</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Pihan et al. 2003</td>
<td>Cervix</td>
<td>Control</td>
<td>35</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>~17%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIC</td>
<td>42</td>
<td>~54% of cases</td>
<td>~55% of cases</td>
<td>~20% of cases</td>
<td>~60%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIN</td>
<td>45</td>
<td>~30% of cases</td>
<td>~20% of cases</td>
<td>~30% of cases</td>
<td>~30%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>Prostate</td>
<td>Control</td>
<td>43</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>~3%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DCIS</td>
<td>42</td>
<td>~60% of cases</td>
<td>~50% of cases</td>
<td>~30% of cases</td>
<td>~70%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brest</td>
<td>6</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>~0%</td>
<td>NA</td>
</tr>
</tbody>
</table>

Abbreviation: LGIN, low-grade intraepithelial neoplasms; HGfN, high-grade intraepithelial neoplasm; CIC, carcinoma in situ of cervix; PIN, carcinoma in situ of prostate; DCIS, ductal carcinoma in situ.

Definition of centrosome abnormalities (same comment as for first table)
1. Size: more than twice of centrosome diameters in non-tumor cells in the same section or adjacent normal epithelium.
2. Number: > 2 centrosomes per cell
3. Shape: cluster or string-like structure with length > 3μm.
4. Total: the sum of centrosome abnormalities including size, number and shape if any presented
5. Amplification: multiple, irregular, varied sized centrosomes scattered in the cytoplasm.
1.8. FREQUENCY OF CENTROSONE ABNORMALITIES IN HUMAN CANCER TISSUES

Although, the number of studies using paraffin-embedded tissue was small (Table I); all studies reported a wide range of centrosome abnormalities (10-85% of cases) associated with cancer. These results support the general concept that centrosome abnormalities are correlated with clinicopathological features of human cancers including change of tissue architecture, aggressiveness, histological grade and recurrence. In the following sections, I will review and discuss the relationship between centrosome abnormalities and the clinicopathological features of cancers. I will also discuss the limitations in interpreting the results from current studies.

1.8.1. Frequency of centrosome abnormalities and clinicopathological features of cancer

Studies have reported a relationship between the frequency of centrosome abnormalities with different clinicopathological features such as change of tissue architecture, grading, aggressiveness and recurrence of cancer. These are described below.
1.8.1.1. **Centrosome abnormalities and change of tissue architecture in cancer development**

Centrosome abnormalities are expected to affect cell shape, polarity and motility, and could cause changes of tissue architecture because of their association with microtubules. A number of studies have shown that centrosome abnormalities cause tissue architecture changes in cancer. For example, Pihan *et al.*, (2001) studied various grades of prostate cancer, and concluded that centrosome dysfunction contributed to the progressive loss of cellular and glandular architecture in prostate cancer. In another study, high grade breast cancer cells show a high frequency of loss of structural differentiation, increased microtubule organization, and centrosome abnormalities (Lingle *et al.*, 1998).

1.8.1.2. **Frequency of centrosome abnormalities correlate with the grade and aggressiveness of cancer**

Correlation of increasing frequency of centrosome abnormalities with increasing grade or aggressiveness of cancer has been reported in several papers. A study conducted by Hsu and co-workers shows a progressive increase in centrosome amplification with advancing tumor stages in human ovarian cancers: centrosome amplification was observed in 61% of stage I cancer (n = 23), 83% of stage II cancer (n = 30) and 100% of stage III cancer (n = 10) (Hsu *et al.*, 2005). In another study investigating the human gall bladder system, a similar finding has also found: a higher frequency of centrosome abnormalities in biliary malignancies at advanced stages (III-IV) compared to cancers at earlier stages (I-II) \( P < 0.001 \) (Kuo *et al.*, 2000). Finally a correlation between histological progression and
centrosome abnormalities has also been reported for the prostate. Prostate cancers with high Gleason grades (IV-V) show a 1.3-1.4-fold elevation in frequency of cells with elevated centrosome number and centrosome size compared to prostate cancers with low Gleason grades (II-III) (Pihan et al., 2001).

I.8.1.3. **Frequency of centrosome abnormalities correlate with cancer recurrence**

Centrosome abnormalities have also been correlated with tumor recurrence. In head and neck squamous cell carcinoma, centrosome amplification was more frequently found in cancers that went on to recur (90%, n = 10) compared to cancer without recurrence (37.5%, n = 8). However, this study has only a small sample size (total n = 18) and a short follow-up period (12 months) (Gustafson et al., 2000).

I.8.2. **Limitations in interpreting centrosome abnormalities in current studies**

When interpreting study results, some important limitations should be taken into consideration. These include the lack of reliable control samples, the diverse scoring criteria used in evaluating centrosome abnormalities in the different studies and the lack of information associating with dysplasia lesions.
I.8.2.1. **Lack of control samples**

Among 8 studies, only 2 studies used normal tissues as control samples. And one of the two studies has a very small number of normal control samples ($n = 3$). The study conducted by Nakajima *et al.* (2004) only made a comparison between diploid liver cancer and non-diploid cancer. The study conducted by Kuo *et al.* (2000) used benign diseased samples as controls. The last four studies conducted by Koutsamin *et al.* (2006), Pihan *et al.* (2001), Gustafson *et al.* (2000) and Carroll *et al.* (1999) used adjacent normal epithelium adjacent to the tumor (cancer-adjacent normal) as controls samples. Cancer-adjacent epithelium and benign diseased samples are poor controls samples since genetic change could be present in such control samples, even before dysplastic or cancer phenotype is apparent. Lack of reliable control samples obviously limits confidence in the above studies.

I.8.2.2. **Diverse scoring criteria used in evaluating centrosome abnormalities in paraffin tissues**

To date, there are no standard criteria for evaluating centrosome abnormalities. Among the 8 studies (summarized in Table 1), centrosome abnormalities have been evaluated based on the following criteria:

1. presence/absence of amplification: head and neck cancers and lung cancer
   
   (Carroll *et al.*, 1999; Koutsami *et al.*, 2006)

2. grade of amplification: oral cancer (Gustafson *et al.*, 2000)
(3) size and number: ovary cancer and pancreatic cancer (Sato et al., 1999; Hsu et al., 2005)

(4) size, number and shape: liver cancer, prostate cancer and gall bladder cancer

(Kuo et al., 2000; Pihan et al., 2001)

The lack of standard evaluation criteria makes comparisons between different studies difficult. Furthermore, the study results on centrosome abnormalities are limited if they only report the presence/absence or grades of amplification.

1.8.2.3. Lack of information on association with dysplasia

As shown above there is little information on the prevalence of centrosome abnormalities in premalignancies including dysplasia. Even among the two papers shown in Table 2, little information is given that correlates centrosome abnormalities with histology, e.g. where the abnormalities are in relation to epithelial cell layers and degree of dysplasia. Such information is critical to support the hypothesis that centrosome abnormalities are a driving force of carcinogenesis.
I.9. FREQUENCY OF CENTROSOOME ABNORMALITIES IN ORAL CANCER

There are only two papers published on centrosome abnormalities in human oral SCC samples. However, both studies have small sample sizes (Carroll et al., 1999; Gustafson et al., 2000). In the study by Gustafson et al. (2000), centrosome amplification was found in 17 of 18 oral cavity SCCs. The study conducted by Carroll et al. (1999) examined 12 cases of squamous cell carcinoma of the head and neck. Eight of 12 cases showed positive centrosome amplification. In each study, the nature of the centrosome abnormalities is not described. Based on these studies, a high frequency of centrosome abnormalities exists in oral cancers. Hence, further studies conducted to obtain more detailed information are warranted.
1.10. FREQUENCIES OF CENTROSome ABNORMALITIES IN PRE CANCERS

1.10.1. Animal models

In animal models, centrosome abnormalities have been found at early stages of carcinogenesis and have been shown to increase in severity with the progression of the lesions. In the rat mammary carcinogenesis model using female Wistar Furth rats treated with methyl-nitrosourea, numerical changes of centrosomes (> 2 centrosomes per cell) were observed at the dysplasia stage and shown to progress over time (Goepfert et al., 2002). In the female August Copenhagen Irish rat model, employing treatment with 2 or 3 mg 17β estradiol, centrosome amplification was first found in 30% of cells with focal dysplasia. This frequency increase as the lesions progressed to ductal carcinoma in situ (38%) and invasive carcinoma (90%) (Li et al., 2004).

1.10.2. In human tissue samples

There is a marked lack of information on centrosome abnormalities in preinvasive lesions in human tissues. There are only two studies using human precancers, which are summarized in Table 2. The study conducted by Pihan et al., (2003) assess centrosome abnormalities (number, size, shape) in carcinoma in situ of cervix, breast and prostate, late stage of precancerous lesions. Centrosome abnormalities were observed in 62% of CIS in the cervix, 75% in breast and 28% in prostate. The study conducted by Keyser et
*al.* (2005) investigated centrosome abnormalities (number and intensity of γ-tubulin stained signals) in human colon tissues. The mean centrosome signal per cell differ significantly ($P < 0.0001$) between the normal colonic epithelium (0.88) and dysplasia/cancer tissue (low-grade dysplasia 1.79; high-grade dysplasia 2.26; invasive adenocarcinoma 2.27) (Kayser *et al.*, 2005). Interestingly, the numerical and structural centrosome alterations observed during the transition of normal colonic epithelium to low-grade dysplasia did not progress further along the adenoma–carcinoma sequence, remaining equally elevated in high-grade dysplasia and invasive adenocarcinomas.

No study has investigated centrosome abnormalities using human OPLs.
II. STATEMENT OF PROBLEMS

Oral SCC is believed to progress through hyperplasia to increasing degrees of dysplasia (mild, moderate and severe) to CIS and finally to invasive SCC. Once SCC is formed, the prognosis is dismal. Its 5-year survival rate has been steady at 30% to 50% without significant improvement in the past three decades. The poor prognosis is largely a result of late diagnosis. The key to improving the 5-year survival rate is early detection of early-stage oral SCC and identification of the small percentage of oral premalignant lesions (OPLs) at high-risk for cancer progression.

The current gold standard for cancer risk assessment of OPLs is histology involving assessment of the presence and degree of dysplasia. However, while histology is a good risk indicator for high-grade lesions (severe dysplasia and CIS), it is a poor indicator of cancer risk for the majority of OPLs with only low-grade dysplasia. Development of new markers that can identify high-risk OPLs is highly desired.

Genomic instability (GI) is now believed by most to be a driving force of cancer development. CIN is the most common type of GI and has the most serious impact on cells due to the huge gains and/or losses of chromosomes. CIN has been found in all types of human cancers including oral cancer and oral precancer. Using techniques of FISH, array CGH and genome-wide allelotyping for LOH, wide-spread CIN have been
found in both oral cancer and oral precancer. However, the mechanisms producing such changes are still unknown.

Centrosome abnormalities have long been hypothesized to play an important role in CIN through production of multipolar mitosis and asymmetrical segregation of chromosomes. With the current interest in the study of GI and CIN, and with recent findings of roles of centrosome in cell cycle regulation and tissue architecture regulation, studies of centrosome abnormalities are gaining attention in cancer research. Numerical and structural alterations to centrosomes have been investigated in a limited number of studies in human cancer, including only two studies on oral SCC, because of technical difficulties in using human tissues. Most of these studies had limitations because of lack of reliable controls or small sample sizes. Few have studied the size, shape and number of centrosome abnormalities using paraffin tissue sections. This makes it difficult to determine relationships among these centrosome abnormalities. None of these studies on human samples have investigated the presence/absence of centrioles in abnormally amplified centrosomes.

There is a marked lack of study of centrosome abnormalities in precancerous lesions. Up to date, there are only two studies on human tissues - one on CIS (prostate, cervix, and breast) the last stage before invasion, the other using glandular epithelium of the colon. There is no study on centrosome abnormalities in OPLs or any human squamous dysplasia. The study of centrosome abnormalities in OPLs is not only important to the
understanding of oral cancer development but also may provide new tool/markers for identifying high-risk OPLs.
III. HYPOTHESIS

Structural and numerical abnormalities of centrosomes exist in both oral cancer and oral premalignant lesions and the frequency of these abnormalities correlates with histological grades during oral cancer progression.

If the data support this hypothesis, it would suggest that centrosome abnormalities play a role in oral carcinogenesis and might be the driving force of chromosomal instability.
IV. OBJECTIVES

1. To compare the patterns of numerical and structural centrosome abnormalities (size, number and shape patterns) in oral hyperplasia, dysplasia (mild, moderate and severe dysplasia and CIS), and invasive SCC by means of fluorescence immunostaining analysis with mouse monoclonal anti-γ-tubulin antibody.

2. To determine and compare the patterns of centrioles in oral hyperplasia, dysplasia (mild, moderate and severe dysplasia and CIS), and invasive SCC by means of fluorescence immunostaining analysis with rabbit polyclonal anti-α/β-tubulin antibody.

3. To determine and compare the relationship among above assessed centrosome abnormalities using serial tissue sections of above samples sets.
V. MATERIALS AND METHODS

V.1. PATIENTS AND SAMPLES

Archival oral biopsy samples were selected from patients that attended the Oral Mucosa Disease Clinics at the UBC Hospital and Vancouver General Hospital between 1997 and 2005. The two clinics are referral centres in the province British Columbia for the diagnosis and management of oral mucosa diseases including oral premalignant and malignant lesions. These patients were primarily referred by community dentists.

V.1.1. Selection criteria for the study samples

The following selection criteria were used for the study samples:

(1) The histological diagnosis was normal, hyperplasia, dysplasia, CIS and SCC. The histopathological diagnosis was confirmed by Dr. Catherine Poh, an Oral Pathologist at the BC Provincial Oral Biopsy Service using the criteria established by the World Health Organization (WHO, 1978).

(2) Confirmation that the samples were sufficiently large enough for at least 20 sections with thickness of 4μm for the study.

(3) Availability of pathological reports for review.
V.1.2. Sample sets

Selected slides were categorized into 5 groups based on the histological diagnosis:

1. Group 1: 12 normal oral mucosa.
2. Group 2: 4 oral hyperplasia.
5. Group 5: 13 invasive oral SCC.

Low-grade dysplasia consists of either mild or moderate dysplasia. High-grade dysplasia includes severe dysplasia and CIS.

Figure 6 presents a flow chart showing the study design and groups of samples used for each experiment in this thesis. All samples were evaluated for centrosome shape and number abnormalities. Size measurement was technically challenging. Only a fraction of the samples gave clear enough signals for computer analysis and the procedure was very labour intensive. Hence, only a subset of samples representing various histological stages was analyzed. Similarly, assessment of samples for the presence/absence of centrioles was restricted to those samples that showed amplified centrosomes (i.e., multiple, irregular-shaped and/or varied sized centrosomes scattered in the cytoplasm). Finally, assessment of normal bipolar mitosis and abnormal multipolar mitosis were only conducted on those samples with sufficient identifiable mitotic cells (defined as > 2 identifiable mitotic cells in normal oral mucosa and hyperplasia; > 10 identifiable mitotic cells in low-grade dysplasia, high-grade dysplasia and oral SCC). Mitotic figures are
infrequent in most sections and are even less frequent within a visual plane. Hence, a subset of 18 cases (3 normal oral mucosa, 2 oral hyperplasia, 8 oral dysplasia and 5 invasive oral SCC) was chosen from the 50 samples due to the lack of identifiable mitotic cells on the other samples.
Figure 6: Flow chart for the study design and groups of samples for each experiment
V.1.3. **Patient information**

The following patient information was collected for each case: age, gender, and anatomical site of the oral lesion. No specific patient identifiers were provided to ensure patient confidentiality. The study protocols were reviewed and accepted by the Research Ethics Board, Simon Fraser University and were consistent with accepted standards.

V.2. **SLIDE PREPARATION**

Serial 4μm-thick sections were cut from each paraffin-embedded tissue block by using a microtome. One slide was submitted for haematoxylin and eosin (H&E) staining for reference and the remaining slides were processed for indirect fluorescence immunostaining.

V.3. **HAEMATOXYLIN AND EOSIN (H & E) STAINING**

Slides were baked at 37°C overnight in an oven, then at 60 to 65°C for 1 hour before being placed at room temperature to cool. Samples were deparaffinized by two changes of xylene for 15 minutes followed by a graded alcohol series (100%, 95%, and 70% ethanol), and then rehydrated by rinsing in tap water. Slides were then placed in Gill’s Haematoxylin for 5 minutes, followed by a tap water rinse, and were then blued with 1.5% (w/v) sodium bicarbonate. After rinsing in water, slides were counterstained with eosin, dehydrated, and mounted for coverslipping. This step was performed by technician, Vennie Chou.
V.4. FLUORESCENCE IMMUNOSTAINING

Slides were baked in an incubator at 65°C for 6 hours. Tissue sections were then deparaffinized with 3 ten-minute xylene soakings. Xylene was removed with 2 three-minute soakings in 100% ethanol, followed by rehydration of samples using a graded alcohol series (100%, 95%, 80%, 70% and 50% ethanol) and 2 changes of distilled deionized water (ddH₂O). After 3 changes of Phosphate Buffered Saline (PBS), the samples were ready for antigen retrieval.

V.4.1. Antigen retrieval (AR)

Different antigen retrieval methods were tested prior to running study samples. The test methods include heat-induced AR in different solutions [e.g. 0.2mM EDTA, citrate buffer pH 6.0 and 0.5% triton-X/tris buffered saline (TBS)], enzyme induced AR methods (proteinase K) and a combination of both methods in a microwave or a steamer. All AR methods showed similar staining patterns but the combination of citrate buffer pH 6.0 followed by the 5μg/ml proteinase K yielded the best staining results.

A staining jar containing citrate buffer (pH6.0) was placed within a steamer (T-Fal Steam Cuisine Compact - VC1001, T-fal Inc.) and was preheated for at least 15 minutes to an equilibrium temperature of 98°C. Samples were immersed in the staining jar and incubated for 5 minutes. The jar containing the specimens was removed from the steamer and allowed to cool down at room temperature for 30-45 minutes. After rinsing
with 5µg/ml proteinase K in 1mM CaCl₂/TBS, the sections were incubated with 500µl of 5µg/ml proteinase K in 1mM CaCl₂/TBS in a 37°C incubator for 30-100 minutes. The samples were then washed three times in PBS. This was followed by pre-incubation with a blocking solution (consisting of 10% goat serum, 3% bovine serum albumin (BSA) and 0.3% Tween-20 in PBS) at room temperature for one hour. A moist chamber was used for this step to prevent the sections from drying out.

**V.4.2. Indirect fluorescence immunostaining with different antibodies**

**V.4.2.1. Anti-γ antibody**

A mouse monoclonal anti-γ tubulin antibody (clone GTU-88; Sigma) was used to detect the pericentriolar matrix of the centrosomes, highlighting the whole centrosome area and allowing for measurement of size, number and shape alterations to centrosomal regions. Anti-γ antibody was diluted (1:300 dilution) in PBS containing 0.3% Tween-20 and 3% BSA. This solution was applied to the tissue sections and incubated at 4°C overnight.

One negative control slide was prepared at the same time by omitting the secondary antibody. The next day, slides were washed with PBS containing 0.3% Tween-20 (PBS-T) three times. The antigen-antibody complexes were detected by applying a secondary fluorescent antibody (Alexa Fluor 488 goat anti-mouse IgG antibody; Molecular Probes; 1:300 dilution; excitation wave length 494nm; emission wave length 517nm) in PBS containing 0.3% Tween-20 and 3% BSA at 4°C for 45 minutes. Samples were
subsequently washed well with PBS-T (PBS containing 0.3% Tween-20) and PBS, air-dried and mounted with a mixture of 4’, 6-diamidino-2-phenylindole (DAPI; 32-404830; Vysis) and slowfade gold anti-fade reagent (S36936; Molecular Probes) to counter-stain the nuclei. The antibody-labelled components fluoresced green. The slides were stored at -20°C until analysis.

V.4.3. Co-immunostaining with anti-γ tubulin antibody and anti-α/β tubulin antibody

Studies have shown that some abnormal centrosomes do not contain centrioles (Pihan et al., 1998). In this study, 11 cases that showed amplified centrosomes (multiple, irregular, varied sized, stained γ-tubulin signals scattered in the cytoplasm) using anti-γ tubulin antibody were further analyzed by co-immunostaining with anti-α/β tubulin antibody.

Multipolar mitosis is a common feature that coexists with centrosome abnormalities. To evaluate the frequency of multipolar mitosis and normal bipolar mitosis, another subset of 18 cases were selected from the 50 samples in the sample set based on the numbers of identifiable mitotic cells.
A rabbit polyclonal anti-α/β-tubulin antibody (# 2148; Cell Signalling) that detects the mother centrioles, daughter centrioles and mitotic spindles was used. This probe signal was red after complexing with Alexa Fluor 555 goat anti-rabbit antibody (excitation wave length 556nm; emission wave length 573nm), and was used to highlight the centrioles during interphase, and mitotic spindles during mitosis.

Both anti-γ and anti-α/β tubulin antibodies were diluted (1:300 dilution for both antibodies) in PBS containing 0.3% Tween-20 and 3% BSA. The antibody solution was applied to the tissue sections and incubated at 4°C overnight. Two negative control slides were prepared at the same time by omitting the anti-γ tubulin antibody and omitting both primary antibodies. Samples were washed with PBS containing 0.3% Tween-20 (PBS-T) three times. The antigen-antibody complexes were detected by applying two secondary fluorescent antibodies purchased from Molecular probes (Alexa Fluor 488 goat anti-mouse IgG antibody and Alexa Fluor 555 goat anti-rabbit IgG antibody; Molecular Probes; 1:300 dilution for both antibodies) in PBS containing 0.3% Tween-20 and 3% BSA at 4°C for 45 minutes. Sections were subsequently washed with PBS-T and PBS intensively, air-dried and mounted with a mixture of 4′, 6-diamidino-2-phenylindole (DAPI; 32-404830; Vysis) and slowfade gold anti-fade reagent (S36936; Molecular Probes) for counter-staining nuclei. The slides were stored at −20°C until analysis.
V.5. EVALUATION OF CENTROsome ABNORMALITIES

V.5.1. Evaluation of centrosome (γ-tubulin stained signals) abnormalities

Centrosomes (anti-γ tubulin stained signals) were evaluated for three different alterations: size, shape and number of centrosomes. Size measurement was made by using Image Pro computer software (Version 6.0, Media cybernetics, MO, USA) on a computer imaging system that was attached to an Olympus BX-61 fluorescence microscope. Shape and number changes were evaluated manually using the same microscope.

V.5.1.1. Size measurement using a computer imaging system

The size measurement of centrosomes (γ-tubulin signals) was made utilizing a computer imaging system. Under 1,000X magnification, 5 frames of 1μm interval z-stacked images were acquired using QED in vivo image software (Version 3.0; Media Cybernetics, MO, USA) and a monochrome camera (Evolution QEi; Media Cybernetics, MO, USA) (see figure 7). On average, 20 to 40 sets of Z-stack non-overlapping images were taken for each case. Images were saved and analyzed using Image Pro software (Version 6.0, Media cybernetics, MO, USA). Prior to making any measurements, images were digitally zoomed in to 200%. The sharpness of each centrosome signal was examined carefully on all five frames. For each centrosome, the image with the sharpest signal was selected from all five frames and then measured. Measurements were made by visually identifying the two most extreme points on the outer perimeter of each...
centrosome, marking them and then using the software to measure the length of the line created between the two points (Figure 7). A minimum of 400 cells/case in normal and oral dysplasia (due to smaller sample sizes in these categories) and 500 cells in oral cancers (tended to be larger sample sizes) were measured manually. In sections with marked amplification of centrosomes, only cells with 1 or 2 stained γ-tubulin signals were measured to eliminate the influence of changes of centrosome numbers on size.
Figure 7: Manual size measurement of centrosomes

Under 1,000X magnification, 5 frames of 1μm interval Z-stack images were acquired. Images were saved and analyzed by Image Pro software. The sharpness of each centrosome signal was examined carefully on all five frames. Only the sharpest signal of each centrosome from the five frames was selected and then measured. In this example, Frame 3 and 4 were used for the specific anti-γ tubulin signal (see orange arrow). Manual size measurement was made by identifying the two most extreme points on the outer perimeter of each sharply focused centrosome and then using software to measure the linear length created between the two points. The orange arrow points to the same two centrosomes present on all 5 frames. Original magnification: 1,000X.
Cases with centrosome size measured

Because size measurements were very labour intensive (each sample took at least 20 hours to analyze their images), only a subset of the study cases had their centrosome size measured. The following cases were randomly selected from the study groups for centrosome size measurement: 9 normal oral mucosa, 2 oral dysplasia (one low-grade and one high-grade) and 4 oral SCC.

V.5.1.2. Shape and number scoring under the fluorescence microscope

Centrosome profiles (γ-tubulin signals) were examined for shape and number changes in all cases in the study set (see section V.1.2) using visual analysis of the samples with the Olympus BX-61 fluorescence microscope. A minimum of 500 cells from each section were scored under 1,000 X magnification. Each cell was scored only once. Only the area with non-overlapping cells were chosen carefully for scoring. Each cell was classified into one of the four shape/number abnormality categories.

(1) Abnormal shape
   a String-like structure: multiple stained γ-tubulin signals were organized as string-like structures with estimated lengths exceeding approximately 3μm.
   b Cluster: multiple stained γ-tubulin signals were aggregated as a patch pattern and the number could not be scored.
(2) Abnormal number

a  More than two stained $\gamma$-tubulin signals: more than two stained $\gamma$-tubulin signals were found in a localized area of cytoplasm without being clustered, and with their associated cell border not overlapping any other surrounding cell.

b  Amplification: multiple, irregular, varied sized, stained $\gamma$-tubulin signals scattered in the cytoplasm.

Representative samples showing these centrosome abnormalities are presented in Figure 8.
Figure 8: Centrosome profile scoring criteria. Abnormal centrosome profiles were classified into 3 different categories of size (B), shape (C) and number (D).

Centrosomes were stained with anti-γ tubulin antibody (green) and nuclei were counterstained with DAPI (blue). (A) A normal centrosome pattern containing two uniform dot-like, γ-tubulin-stained signals in a cell. Abnormal centrosome profiles (B-D): (B) Size: (b-1) showing an enlarged centrosome. (C) Shape: (c-1) showing a string-like structure formed by numerous centrosomes aggregation, with length > 3μm; (c-2) showing a cluster with numerous, uncountable aggregated centrosomes. (D) Number: (d-1) showing more than 2 centrosomes in a localized area of cytoplasm with a countable number of centrosomes; (d-2) showing amplification with multiple and varied sized centrosomes scattered in the cytoplasm. Original magnification, 1,000X.
V.5.2. Evaluation of centrosomes (γ-tubulin stained signals), centrioles (α/β tubulin stained signals), and mitotic spindles (α/β tubulin stained signals)

V.5.2.1. Presence/absence of centrioles in amplified centrosomes

Each normal centrosome should contain a pericentriolar matrix and 2 centrioles (Figure 9A). When cells have abnormally amplified centrosomes, these amplified centrosomes may or may not have amplified centrioles. In this study, amplified centrosomes were categorized into two groups based on the numbers of centrioles in each cell:

1. amplification of acentriolar centrosomes: the number of α/β-tubulin stained signals (centrioles) is 1 or 2 only while the number of stained γ-tubulin signals (centrosomes) is more than 2 in one cell (Figure 9B)
2. amplification of centrosomes with an excess number of centrioles: the numbers of both α/β-tubulin stained signals (centriole) and γ-tubulin stained signals (centrosomes) are more than 2 in one cell (Figure 9C)

Each cell with amplified centrosomes was scored only once. In oral dysplasia and oral SCC, only cells with amplified centrosomes in the diseased areas were scored. Only a subset of the total sample set containing severe amplified centrosomes (multiple irregular, varied sized centrosomes scattered in the cytoplasm) was studied with this protocol. The subset included: 3 normal oral mucosa, 5 dysplasia (2 low-grade and 3 high-grade) and 3 SCC.
Figure 9: Centrosome/centriole profile scoring criteria. Abnormal centrosome profiles are classified into 2 different categories of amplification of acentriolar centrosomes and centriolar centrosomes.

The centrosome and centriole were stained with antibodies to γ-tubulin (green) and α/β tubulin (red) respectively. Nuclei were stained with DAPI (blue). (A) Normal pattern of centriolar centrosomes have two green dots (centrosomes; γ-tubulin stained signals) overlapping with two red dots (centrioles; α/β-tubulin stained signals). (B) Amplification of acentriolar centrosomes. Only 2 centrioles (red dots) coexist with numerous centrosomes (green γ-tubulin stained signals) in one cell. (C) Amplification of centriolar centrosomes. Multiple centrosomes (green dots) and centrioles (red dots) co-localized in one cell. (Original magnification, 1,000 X).

V.5.2.2. Evaluation of bipolar and multipolar mitosis

A different subset of samples was selected for evaluating the frequency of bipolar and multipolar mitosis using the fluorescence microscope. These samples were chosen based on the availability of identifiable mitotic cells in each tissue section (> 2 identifiable mitotic cells in normal oral mucosa and hyperplasia; > 10 identifiable mitotic cells in low-grade dysplasia, high-grade dysplasia and oral cancers). This subset included: 3 normal samples, 2 hyperplasia, 8 with dysplasia (4 low-grade and 4 high-grade) and 5
SCC. All mitotic cells were evaluated based on the number of mitotic spindles. Only mitotic cells with clearly 2 or more than 2 mitotic spindles were counted. In oral hyperplasia, dysplasia and cancer tissues, only the diseased areas were evaluated (see example in Figure 10).

A: Bipolar mitosis
B: Multipolar mitosis

Figure 10: Normal bipolar mitosis and multipolar mitosis

The centrosomes and mitotic spindles were stained with antibodies to $\gamma$-tubulin (green) and $\alpha/\beta$ tubulin (red) respectively. Nuclei were counter-stained with DAPI (blue). (A) A normal mitotic cell with bipolar mitotic spindles (red color). (B) An abnormal mitotic cells with multipolar (tripolar) mitotic spindles (red color).
V.6. STATISTICAL ANALYSIS

Sample means and standard deviations were determined for centrosome abnormalities of size, shape and number. Box plots were used to display the following values:

1. median: horizontal bar inside the box
2. 25\textsuperscript{th} percentile of the data distribution: lower hinge of the box
3. 75\textsuperscript{th} percentile of the data distribution: upper hinge of the box
4. minimum value: \( \text{\textdagger} \) below the box
5. maximum value: \( \text{\textdaggerdbl} \) above the box

A relative frequency distribution was used to display the size change of centrosomes. This approach showed the mode of the population as well as relative frequency of different centrosome sizes. It was also used to give a visual comparison of frequency distributions in different histological groups. Group comparisons were made with analysis of variance between groups (ANOVA) and Tukey HSD multiple comparisons each utilizing SPSS 13.0 software (SPSS Inc; IL, USA). \( P < 0.05 \) was considered statistically significant.
VI. RESULTS

VI.1. CLINICOPATHOLOGICAL FEATURES OF PATIENTS

Table 3 summarizes clinicopathological features of the patients included in this study, showing average age and gender (% male) in relationship to histological diagnosis. The mean age for the entire study group is $53 \pm 13$ years, with similar ages for individuals in each histological grouping. Males account for 49% of all cases with gender distribution being similar across histological groups. There were a total of 33 cases (66%) biopsied from high-risk areas including the tongue, palate and the floor of the mouth. The percentage of high risk sites are higher in low-grade dysplasia (80%), high-grade dysplasia (67%) and oral cancers (100%) compared to the normal tissue (3%) and hyperplasia (25%).

Table 3: Demographic features of patients

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Normal</th>
<th>HYP</th>
<th>LGD</th>
<th>HGD</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>50</td>
<td>12</td>
<td>4</td>
<td>15</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Mean age (year ± SD)</td>
<td>$53 \pm 13$</td>
<td>$47 \pm 15$</td>
<td>$52 \pm 9$</td>
<td>$55 \pm 14$</td>
<td>$53 \pm 6$</td>
<td>$58 \pm 14$</td>
</tr>
<tr>
<td>Proportion male (%)</td>
<td>19/50 (49%)</td>
<td>5/12 (42%)</td>
<td>2/2 (50%)</td>
<td>8/15 (53%)</td>
<td>4/6 (67%)</td>
<td>7/13 (54%)</td>
</tr>
<tr>
<td>Anatomical site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case number of high risk site (%)</td>
<td>33 (66%)</td>
<td>3 (25%)</td>
<td>1 (25%)</td>
<td>12 (80%)</td>
<td>4 (67%)</td>
<td>13 (100%)</td>
</tr>
</tbody>
</table>

Abbreviations: HYP, hyperplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia; SCC, squamous cell carcinoma.

High risk sites include tongue, palate and floor of the mouth.
VI.2. CENTROSOME SIZE (DIAMETER) AND HISTOLOGY

Table 4 reports the centrosome diameters for 15 cases, showing the average centrosome size for each case and the histology stage. Table 5 shows mean values for each histological group. Individual values ranged from 0.44 to 0.75μm. The mean centrosome diameter of normal squamous epithelial cells (0.46 ± 0.08μm) was similar to that of the low-grade dysplasia (0.45 ± 0.07μm); it increased in high-grade dysplasia (0.54 ± 0.14μm) with a further increase in SCC (0.71 ± 0.26μm). Analysis using a Tukey HSD comparison showed a significant difference in centrosome size when comparing oral SCC (0.71 ± 0.26μm) with normal oral mucosa (0.46 ± 0.08μm) (P < 0.001).

We chose 0.64μm as a cutoff for ‘normal’ and ‘abnormal’ centrosome size, because in our study, 95% of normal cells had a centrosome size equal to or less than this value. Table 4 shows the percentage of cells with centrosome size greater than this cutoff value for individual cases. Table 5 shows the percentage of cells with increased centrosome size in different histological groups. Both normal and low-grade dysplasia contain less than 5% of cells with enlarged centrosome size compared with 23% of cells in high-grade dysplasia and 55% in invasive SCC. Analysis of variance indicated that there was a significant difference between histological groups and frequency of cells with enlarged centrosomes (> 0.64μm) (P < 0.001) (Table 5).
Table 4: Centrosome size in 15 individual cases

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Total # of cells</th>
<th>Mean diameter of centrosomes ± SD</th>
<th># of cells with centrosomes diameter &gt; 0.64µm</th>
<th>% of cells with centrosomes diameter &gt; 0.64µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>Normal</td>
<td>402</td>
<td>0.44 ± 0.07</td>
<td>10</td>
<td>2.5%</td>
</tr>
<tr>
<td>Case 2</td>
<td>Normal</td>
<td>407</td>
<td>0.46 ± 0.07</td>
<td>17</td>
<td>4.2%</td>
</tr>
<tr>
<td>Case 3</td>
<td>Normal</td>
<td>406</td>
<td>0.47 ± 0.11</td>
<td>47</td>
<td>11.6%</td>
</tr>
<tr>
<td>Case 4</td>
<td>Normal</td>
<td>400</td>
<td>0.49 ± 0.11</td>
<td>49</td>
<td>12.3%</td>
</tr>
<tr>
<td>Case 5</td>
<td>Normal</td>
<td>404</td>
<td>0.45 ± 0.06</td>
<td>13</td>
<td>3.2%</td>
</tr>
<tr>
<td>Case 6</td>
<td>Normal</td>
<td>423</td>
<td>0.45 ± 0.05</td>
<td>7</td>
<td>1.7%</td>
</tr>
<tr>
<td>Case 7</td>
<td>Normal</td>
<td>400</td>
<td>0.47 ± 0.08</td>
<td>20</td>
<td>5.0%</td>
</tr>
<tr>
<td>Case 8</td>
<td>Normal</td>
<td>413</td>
<td>0.45 ± 0.05</td>
<td>1</td>
<td>0.2%</td>
</tr>
<tr>
<td>Case 10</td>
<td>Normal</td>
<td>412</td>
<td>0.45 ± 0.06</td>
<td>10</td>
<td>2.4%</td>
</tr>
<tr>
<td>Case 25</td>
<td>LGD*</td>
<td>415</td>
<td>0.45 ± 0.07</td>
<td>11</td>
<td>2.7%</td>
</tr>
<tr>
<td>Case 17</td>
<td>HGD*</td>
<td>401</td>
<td>0.54 ± 0.14</td>
<td>91</td>
<td>22.7%</td>
</tr>
<tr>
<td>Case 38</td>
<td>SCC*</td>
<td>508</td>
<td>0.75 ± 0.31</td>
<td>286</td>
<td>56.3%</td>
</tr>
<tr>
<td>Case 39</td>
<td>SCC*</td>
<td>507</td>
<td>0.67 ± 0.24</td>
<td>247</td>
<td>48.7%</td>
</tr>
<tr>
<td>Case 40</td>
<td>SCC*</td>
<td>506</td>
<td>0.74 ± 0.27</td>
<td>317</td>
<td>62.7%</td>
</tr>
<tr>
<td>Case 41</td>
<td>SCC*</td>
<td>508</td>
<td>0.66 ± 0.20</td>
<td>269</td>
<td>53.0%</td>
</tr>
</tbody>
</table>

* LGD, low-grade dysplasia; HGD, high-grade dysplasia; SCC, squamous cell carcinoma.
# numbers.

Table 5: Centrosome size and histology

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>LGD*</th>
<th>HGD*</th>
<th>SCC*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td># of cases</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td># of cells</td>
<td>3667</td>
<td>415</td>
<td>401</td>
<td>2029</td>
<td></td>
</tr>
<tr>
<td># of signals</td>
<td>5074</td>
<td>596</td>
<td>517</td>
<td>2303</td>
<td></td>
</tr>
<tr>
<td>Mean diameter (µm)</td>
<td>0.46 ± 0.08</td>
<td>0.45 ± 0.07</td>
<td>0.54 ± 0.14</td>
<td>0.71 ± 0.26</td>
<td></td>
</tr>
<tr>
<td># of cells with centrosomes diameter &gt; 0.64µm</td>
<td>174</td>
<td>11</td>
<td>91</td>
<td>1119</td>
<td></td>
</tr>
<tr>
<td>% of cells with centrosomes diameter &gt; 0.64µm</td>
<td>4.7%</td>
<td>2.7%</td>
<td>22.7%</td>
<td>55.2%</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* LGD, low-grade dysplasia; HGD, high-grade dysplasia; SCC, squamous cell carcinoma.
# numbers.
P values: trend in percentage of cells with enlarged centrosomes (> 0.64µm) across histological groups.
For better visualization, Figure 11 and 12 show frequency distributions and box-plot displays of data for centrosome sizes in different histological groups. As shown in figure 11, the frequency distribution plots, there was a shift in the mode in high-grade dysplasia (0.46µm) and invasive SCC (0.45µm) when compared to normal tissues (0.43µm) and low-grade dysplasia (0.41µm). A positively skewed distribution was apparent in all groups, with the skew more prominent among high-grade dysplasia and oral cancer samples. Figure 12 shows box plots of the same data. Box plots are better indicators of skewed data, visually presenting median values, 25th and 75th percentiles, minimum and maximum values and outliers. There was an increase in median centrosome diameters and in the distribution range with histological progression. Range of centrosome diameters was similar for normal tissue (0.32-1.23µm), low-grade dysplasia (0.32-0.98µm) and high-grade dysplasia (0.26-1.16µm) with an increase for invasive SCC (0.32-2.58µm). The comparison of invasive SCC with normal tissues was significant ($P < 0.001$).
Figure 11: Relative frequency distribution of centrosome sizes of samples from the different histological groups

Relative frequency distribution of centrosome sizes in all four histological groups is positively skewed. (A) Distribution of diameters of 5,075 γ-tubulin stained signals from 3,667 cells in normal tissues (mode, 0.43μm). (B) Distribution of diameters of 596 γ-tubulin stained signals from 415 cells in low-grade dysplasia tissue (mode, 0.41μm). (C) Distribution of 517 γ-tubulin stained signals from 401 cells in one high-grade dysplasia tissue (mode, 0.46μm). (D) The distribution of 2,303 γ-tubulin stained signals from 2,029 cells in oral cancer tissues (mode, 0.45μm).
Figure 12: Box plot analysis for size (diameter) changes of centrosomes in individual cases

(A) Summary of size change of centrosomes in each histological group. The centrosome diameters increase as the disease progresses. (B) Size distribution of centrosomes of individual cases. Centrosome diameters increase in high-grade dysplasia and oral cancer tissues but not low-grade dysplasia. The red horizontal line indicates the 0.64μm centrosome size used for the cutoff of ‘normal’ and ‘abnormal’ (i.e. enlarged) centrosomes. N, normal; LGD, low-grade dysplasia; HGD, high-grade dysplasia, SCC, squamous cell carcinoma. — inside the box, mean of centrosome diameter. 25th percentile, lower hinge of the box. 75th percentile, upper hinge of the box. ◦, mild outliers. ◼, extreme outliers.
VI.3. CENTROSOme SHAPE AND HISTOLOGY

Table 6 shows the relationship between abnormal centrosome shapes and histological diagnosis and Figure 13 presents these data in a graphic form. Centrosome shape is compared in three ways: frequency of cells with cluster formation; frequency of cells showing string-like structures, and total frequency of cells with centrosome shape change (i.e., the sum of these two patterns).

A positive relationship is apparent between the frequency of abnormal centrosome clusters and histological grade ($P = 0.001$) with percentage of cells with this type of anomaly increasing from 0% and 0.1% in normal tissues and hyperplasia respectively to 0.5% in low-grade dysplasia, 1.4% in high-grade dysplasia and 1.2% in oral cancer tissues. The frequency of cells with cluster formation is significantly higher in high-grade dysplasia (1.4%, $P = 0.005$) and oral cancer tissues (1.2%, $P = 0.003$) compared to normal tissues (0%).

String-like abnormalities was fairly rare and present at low frequencies (< 0.05%) in all groups except for HGD. The high frequency in the HGD group may be an anomaly since it represents only one case.

The total percentage of abnormally shaped centrosomes includes both the above anomalies: cluster and string-like formations. There is a statistical significance between the total percentage of abnormally shaped centrosomes and histological stage ($P = 0.001$).
It is interesting to note that high-grade dysplasia has the highest percentage of abnormally shaped centrosomes with a drop among SCC cases. However, this drop in frequency may be a result of the combination of small numbers of samples in each group and low frequency of anomalies.

**Table 6: Abnormal centrosome shape and histology**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HYP*</th>
<th>LGD*</th>
<th>HGD*</th>
<th>SCC*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td># of Cases</td>
<td>12</td>
<td>4</td>
<td>15</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Cluster (%)</td>
<td>0.0 ± 0.0%</td>
<td>0.1 ± 0.1%</td>
<td>0.5 ± 0.4%</td>
<td>1.4 ± 1.0%</td>
<td>1.2 ± 1.3%</td>
<td>0.001</td>
</tr>
<tr>
<td>String-like (%)</td>
<td>0.2 ± 0.5%</td>
<td>0.05 ± 0.1%</td>
<td>0.05 ± 0.2%</td>
<td>1.98 ± 3.3%</td>
<td>0.0 ± 0.0%</td>
<td>0.009</td>
</tr>
<tr>
<td>Total shape (%)</td>
<td>0.2 ± 0.5%</td>
<td>0.15 ± 0.2%</td>
<td>0.5 ± 0.4%</td>
<td>3.4 ± 3.6%</td>
<td>1.2 ± 1.3%</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*HYP, hyperplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia; SCC, squamous cell carcinoma.

# numbers.

P values: trend in percentage of cells with abnormally shaped centrosomes across histological groups.
Figure 13: Box plot analysis of abnormal centrosome shape and histology

(A) Cluster formation of centrosomes. (B) String-like structure with length more than 3μm. (C) Total shape changes (i.e. cluster plus string-like abnormalities). All 3 categories show statistical significance. However, the change of string-like structure is not directly associated with histolopathological progression, since it is elevated only in the HGD, dropping to zero in the SCCs. N, normal; LGD, low-grade dysplasia; HGD, high-grade dysplasia, SCC, squamous cell carcinoma. — inside the box, mean of centrosome diameter. 25th percentile, lower hinge of the box. 75th percentile, upper hinge of the box. ⊗, mild outliers. ⊘, extreme outliers.
VI.4. CENTRO SOME NUMBER AND HISTOLOGY

Table 7 shows the relationship between centrosome number and histological diagnosis. Figure 14 presents these data graphically. Centrosome numbers are compared in three ways: frequency of cells with more than 2 centrosomes; frequency of cells showing centrosome amplification, and total frequency of cells with centrosome number change (i.e., the sum of the two patterns). Centrosome amplification is defined as multiple, irregular, varied sized centrosomes scattered in the cytoplasm.

The frequency of cells with greater than 2 centrosomes was significantly associated with increasing histological grades ($P < 0.001$). The frequency of such cells is low in normal tissue (mean value, 0.03%) and in hyperplasia (0.1%) increasing slightly in low-grade dysplasia (0.7%). Frequencies further increased to 1.8% in high-grade dysplasia and 1.3% in oral cancers. Frequencies are statistically significantly higher in high-grade dysplasia and oral cancers compared to normal tissues ($P < 0.001$) and also in high-grade dysplasia, compared to low-grade dysplasia ($P = 0.004$).

There is no significant difference in amplification of centrosomes among the different histological groups. Amplification frequencies are much higher across all histological groups when compared to frequencies of cells with more than 2 centrosomes (range for amplification, 7.7% to 15.3% compared to 0.03% to 1.8% for cells with more than 2 centrosomes). The lack of association of total number changes of centrosome with histology is due to this much higher frequency of change in amplification. It is
interesting to note that the highest level of amplification is observed in normal tissues (15.3%) and not in tissue with histological change. This suggests that the phenomenon called “amplification” is probably not related to carcinogenesis and that it may have an alternate etiology.

Table 7: Frequency of cells showing abnormal centrosome numbers among samples belonging to different histological groups

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>HYP*</th>
<th>LGD*</th>
<th>HGD*</th>
<th>SCC*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td># of samples</td>
<td>12</td>
<td>4</td>
<td>15</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td># of cells &gt; 2 centrosomes (%)</td>
<td>0.03 ± 0.07%</td>
<td>0.1 ± 0.1%</td>
<td>0.7 ± 0.7%</td>
<td>1.8 ± 0.9%</td>
<td>1.3 ± 0.7%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td># of cells with amplification (%)</td>
<td>15.3 ± 30.6%</td>
<td>10.7 ± 21.5%</td>
<td>12.7 ± 23.1%</td>
<td>7.7 ± 8.9%</td>
<td>13.9 ± 26.3%</td>
<td>0.98</td>
</tr>
<tr>
<td>Total # of cells with above anomalies (%)</td>
<td>15.3 ± 30.7%</td>
<td>10.8 ± 21.5%</td>
<td>13.4 ± 22.8%</td>
<td>9.5 ± 9.0%</td>
<td>15.2 ± 26.0%</td>
<td>0.99</td>
</tr>
</tbody>
</table>

*HYP, hyperplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia; OSCC, oral squamous cell carcinoma.

# numbers.

Definition of amplification: multiple, irregular, varied sized centrosomes scattered in the cytoplasm of the cell.

P values: trend in percentage of cells with abnormally numbered centrosomes across histological groups.
Figure 14: Comparisons of frequency of centrosome number abnormalities in different histological groups

Box plot analysis was used to compare changes in centrosome number. (A) Frequency of cells with > than 2 centrosomes in each cell. (B) Amplification of centrosomes (multiple, irregular, varied sized centrosomes scattered in the cytoplasm of the cell. There is a trend towards an increase in cells with more than 2 centrosomes associated with histological progression ($P < 0.001$). In contrast, the frequency of amplification is not associated with alteration to histology ($P = 0.99$) nor is total number change ($P = 0.98$). (C) Frequency for all cells showing numerical changes (i.e., sum of data in A and B). N, normal tissues; HYP, hyperplasia; LGD, low-grade dysplasia; HGD, high-grade dysplasia and SCC, squamous cell carcinoma. — inside the box, mean of centrosome diameter. 25th percentile, lower hinge of the box. 75th percentile, upper hinge of the box. ◦, mild outliers. ◇, extreme outliers.
VI.5. MULTIPLE CENTROSONE ABNORMALITIES (SIZE, SHAPE AND NUMBER) AND HISTOLOGY

The prior sections have presented data showing an association of multiple types of centrosome abnormalities with oral histopathological progression. As a separate analysis, I next determined how a combination of these features would associate with histological progression. In the first such analysis, each case was scored positive if there was a change in size, shape and/or number. As shown in Table 8, there was a strong relationship between the presence of such change, called “any centrosome abnormality” and progression. It was present in 25% of nondysplastic lesions, the majority (67%) of low-grade dysplasia, and all of the high-grade dysplasia and invasive SCC. Furthermore, the presence of multiple centrosome abnormalities in a sample (defined as the presence of more than 1 type of abnormality in a case) was characteristic of dysplastic lesions (especially HGD) and cancer with only one case of nondysplastic lesions containing such change (see Figure 15). The combination of both centrosome number increase (> 2 centrosomes/cell) and cluster formation was present in more than a quarter of low-grade lesions, the majority of high-grade lesions and invasive SCC, but absent in nondysplastic lesions.
Table 8: Correlation between number and shape changes of centrosomes and histology

<table>
<thead>
<tr>
<th></th>
<th># of cases</th>
<th>Non-dys*</th>
<th>LGD*</th>
<th>HGD*</th>
<th>SCC*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td># of cases</td>
<td>50</td>
<td>16</td>
<td>15</td>
<td>6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td># cases with any centrosome</td>
<td>33</td>
<td>4 (25%)</td>
<td>10 (67%)</td>
<td>6 (100%)</td>
<td>13 (100%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>abnormalities (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of cases &gt; 1 type of</td>
<td>23</td>
<td>1 (6%)</td>
<td>5 (33%)</td>
<td>5 (83%)</td>
<td>12 (92%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>centrosome abnormalities (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># of cases with both</td>
<td>19</td>
<td>0</td>
<td>4 (27%)</td>
<td>5 (83%)</td>
<td>10 (77%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>centrosome number increase (&gt; 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>centrosome /cell) and cluster</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>formation (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Non-dys: non-dysplastic tissues (including normal tissues and hyperplasia); LGD, low-grade dysplasia; HGD, high-grade dysplasia; OSCC, oral squamous cell carcinoma.  
# numbers.  
P values: trend in percentage of cases with centrosome abnormalities across histological groups.
Figure 15: Alterations of centrosomes in normal, low-grade dysplasia (LGD), high-grade dysplasia (HGD) and squamous cell carcinoma (SCC) compared to histology

Left panel: slides were stained with H & E (original magnification, 400X). Middle panel: slides were immunostained with anti-γ-tubulin antibody (green) for centrosomes and DAPI (blue) for nuclei (original magnifications, 400X). Right panel (3) High power fields of the white outlined areas in (2) (original magnification, 1,000X). (A) Normal tissue (N). (B) Low-grade dysplasia (LGD). (C) High-grade dysplasia (HGD). (D) Squamous cell carcinoma (SCC).

A-3, showing normal centrosome patterns has 1 or 2 uniform-sized, dot-like γ-tubulin stained signals in normal mucosa. B-3, showing multiple slightly enlarged centrosomes in LGD. C-3, showing multiple irregular shaped and variously sized centrosomes in HGD. D-3, showing a high frequency of centrosome changes with multiple irregular shaped and variously sized centrosomes in SCC.
VI.6. CO-LOCALIZATION OF CENTROSOMES AND CENTRIOLES

Co-immunostaining with antibodies to γ-tubulin and α/β tubulin is used to localize centrosomes and centrioles respectively in the same tissue section. This procedure is shown in Figure 16. The result of this co-localization is to identify centrosomes without (Figure 16B, acentriolar centrosomes) or with centriole (Figure 16C, centriolar centrosomes). A comparison of normal co-localization of centrioles and centrosome signals is given in Figure 16A.

Table 9 compares the frequency of cells showing acentriolar vs. centriolar centrosomes in cases that show amplified centrosome numbers. In over 90% of cases, most of the cells showing centrosome amplification were acentriolar in characteristic (i.e., they had a greater number of centrosome compared to centriole signals). Centriolar centrosome amplification was comparatively rare, present in 0-1% of cells with amplified centrosomes for all histological groups with the exception of SCC. There was a sharp increase in the proportion of centriolar centrosome in SCCs (9%). However, the association of centriolar centrosome frequency with histology was not significant ($P = 0.12$).
**Figure 16: Co-localization of centrosomes (γ-tubulin) and centrioles (α/β-tubulin) in oral tissues**

Paraffin embedded oral tissues were co-immunostained with antibodies to γ-tubulin (green), a well-known centrosome marker and α/β tubulin (red), the main component of centrioles. The nuclei were counter-stained with DAPI (blue).

A, (upper panel) showing normal pattern with 1 or 2 green centrosomes overlapping the same number of centrioles (red). B, (middle panel) showing cells with amplified centrosomes (green) without centrioles (red), called acentriolar centrosomes. C (lower panel) showing a cell with a co-localized amplified centrioles (red) and centrosomes (green), called centriolar centrosomes. Original magnification, 1,000X.
Table 9: Amplification of acentriolar and centriolar centrosomes in oral tissues

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>LGD</th>
<th>HGD</th>
<th>SCC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td># of cases</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Amplification acentriolar centrosomes (%)*</td>
<td>100</td>
<td>99.5</td>
<td>99</td>
<td>91</td>
<td>0.12</td>
</tr>
<tr>
<td>Amplification centriolar centrosomes (%)**</td>
<td>0</td>
<td>0.5</td>
<td>1</td>
<td>9</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* Amplification of acentriolar centrosomes: multiple centrosomes (γ-tubulin stained signals) with 1 or 2 centrioles (α/β tubulin stained signals) in each cell.

** Amplification of centriolar centrosomes: centrosomes (γ-tubulin stained signals) and centrioles (α/β tubulin stained signals) co-localize in each cell suggesting that they are both amplified.

# numbers.

P values: trend in proportions of cells in each category across histological groups.

VI.7. CENTROSOME AND MITOSIS

Figure 17 shows images of normal bipolar and abnormal multipolar mitosis that were observed in this study. A subset of 18 cases was selected based on the number of identifiable mitotic cells. Overall, the percentage of multipolar mitosis ranges from 0-7.2% in different samples. There was a significant increase in the frequency of multipolar mitosis in association with histological progression. The mean percentage of multipolar mitosis is 0% for samples of normal oral mucosa, oral hyperplasia and low-grade dysplasia; there is an increase in high-grade dysplasia (5%) with a further increase in oral SCC (7.2%) (P = 0.045).
Figure 17: Normal bipolar mitosis and abnormal multipolar mitosis in normal and oral cancer tissue

Co-immunostaining of centrosomes (γ-tubulin) and mitotic spindle (α/β-tubulin) showing normal bipolar mitosis (upper panel) in normal tissues and abnormal multipolar mitosis (lower panel) in oral cancer.

Co-immunostaining with antibodies to γ-tubulin (green), a well-known centrosome marker, and α/β-tubulin (red), the main component of mitotic spindles. The nuclei were counter-stained with DAPI (blue). A (upper panel), showing a normal mitotic cell contains two centrosomes (green) and bipolar mitotic spindles (red). B (lower panel), showing an abnormal multipolar mitotic cell containing 3 centrosomes and 3 groups of mitotic spindles (Original magnification, 1,000X)
VII. DISCUSSION

VII.1. CENTROSOME ABNORMALITIES ARE AN EARLY EVENT IN ORAL CARCINOGENESIS

Structural and numerical alterations to chromosomes are characteristic of human cancers and have been acknowledged as a critical step in cancer development. There has been an increasing interest in the mechanisms underlying such chromosomal changes. Studies have supported that defective mitosis could result in chromosomal change. Proteins playing potential roles in the control of mitosis have been identified and the number is still growing (see section 1.5.3 of Introduction). The transfer of this knowledge to clinical samples in order to determine how disruption of mitosis control affects carcinogenesis has been slow. This has been especially true with respect to studies of chromosomal changes in the premalignant stages of cancer. Major barriers to such studies include limited access to tissues for analysis and the need to modify technology to work with available small formalin-fixed paraffin-embedded samples.

This thesis represents the first cancer study conducted on centrosome abnormalities in oral premalignancies and among the first with oral cancers. The results show an association of numerical and structural centrosome abnormalities with oral cancer progression. There is an increase in centrosome abnormalities accompanying histological change in premalignant lesions and cancers. Alterations to centrosome size, shape and number were all found at the dysplasia stage, suggesting a potential role of centrosome abnormalities in the early stages of oral carcinogenesis. This finding is consistent with
the limited number of studies of centrosome abnormalities in animal models and in human premalignant lesions (sees section 1.11 in Introduction). Centrosome abnormalities have been reported to increase with disease progression, to be present in premalignant stages of female rat mammary carcinogenesis models (Goepfert et al., 2002; Li et al., 2004), human intraepithelial colon cancers (one type of precancerous lesion in colon) (Kayser et al., 2005) and human pancreatic adenomas (Sato et al., 1999).

Together, the data suggests that the development of centrosome abnormalities is a gradual process rather than a sudden event in carcinogenesis. The chance of more than 1 type of centrosome abnormalities also increased with increasing histological severity. Abnormal centrosomes would lead to numerical chromosome change through multipolar mitosis and cell cycle dysregulation (Sluder et al., 2004). Abnormal centrosomes also contribute to structural chromosome changes through asymmetrical chromosome alignment during the metaphase (Saunders et al., 2000). The results do not preclude that centrosome abnormalities are a potential driving force for carcinogenesis rather than a downstream consequence of the very large amount of genetic change that is observed in late-stage cancers.

This is the first study to show the distribution of centrosome sizes at different stages of oral carcinogenesis. The measurement of centrosome size using a computer microscopic imaging system is very time and labour intensive (see V.5.1.1. in Methods and Material). As shown in section VI.2 in Results, the average centrosome diameter was significantly elevated in SCCs compared to normal tissues. The latter analysis involves the measurement of centrosome size in 3,667 normal (9 cases) and 2,303 cancer cells (4
cases). Although size distributions are similar in the 9 normal cases examined and the single low-grade dysplasia (mean diameter of 0.46 and 0.45μm, respectively), there is a sharp increase in the high-grade dysplasia studied (0.54μm) with a further increase in the SCCs (0.71μm, \( P < 0.001 \)). This trend of increase in centrosome size with histological progression is more apparent when a cutoff value is used to examine the data (set at the 95\(^{th}\) percentile for normal centrosome sizes). The percent of cells with diameters greater than this cutoff value was 5%, 3%, 23% and 55% for normal, low- and high-grade dysplasia and SCC. This is striking data and requires a confirmation with a larger set of dysplasia.

This observed association of centrosome size with histological progression is consistent with studies in human prostate cancers, where early staged cancers (Gleason grade II and III) reported a 80% increase to centrosome size and late stage cancers (Gleason grade IV and V) reported a 160% increase to centrosome size (Pihan \textit{et al.}, 2001).

When shape changes to centrosomes are examined, the results show a significant association between the frequency of such change and the severity of histological alteration \( (P = 0.001) \). Two types of change are apparent: cluster and string formation. Cluster formation is an early event apparent even in low-grade dysplasia with an increase in high-grade dysplasia and cancer. Another study has also reported a positive association between cluster formation and increasing histological grade in the prostate system (Pihan \textit{et al.}, 2001; Pihan \textit{et al.}, 2003). String-like structures are primarily apparent among our high-grade dysplasia cases and are virtually absent \( (< 0.2\%) \) in other
samples. Other papers that describe such string-like structures deal with human cancer
tissues including prostate (Pihan et al., 2001; Pihan et al., 2003) gall bladder (Kuo et al.,
2000), and lung (Koutsami et al., 2006) and suggest that they could result from
aggregation of numerous centrosomes. However, the mechanism of cluster formation of
centrosomes and their function is still unresolved. The string-like structures of
centrosomes may play a role in oral carcinogenesis or may be just a variant of amplified
centrosomes.

When evaluating number changes of centrosomes, the results show a strong association
between frequency of cells with greater than 2 centrosomes per cell and histological
grade \((P < 0.001)\). This result is expected for high-grade premalignant lesions and
cancers and is consistent with findings from other laboratories with prostate and
prancrease tissues (Sato et al., 1999; Pihan et al., 2001; Pihan et al., 2003). However,
this thesis also presents data on very early premalignant changes. An increase in
frequency of cells with more than 2 centrosomes is already apparent in low-grade
dysplasia when compared with normal tissues \((P < 0.001)\) and significantly increase
between low-grade and high-grade lesions \((P = 0.004)\). It should be noted that these data
may underestimate the actual proportion of cells that show numerical centrosome change.
Sections are only 4 microns thick and some centrosome may lie outside of the plane of
the cut tissue. This is the so called truncation effect.
A second category of numerical alteration, termed amplification, is also apparent in these samples. Amplification is defined as multiple, irregular, varied sized centrosome signals that are scattered throughout the cytoplasm. This pattern is present at comparatively high frequencies (7-15%) in all histological groups, with the highest frequency among normal samples. A further analysis was performed to assess the presence/absence of centrioles in cells showing amplified centrosomes (see section VII.2 in Discussion).

If centrosome anomalies are playing a role in mitotic dysfunction, one would expect to see abnormal multipolar mitosis in OPLs and cancers. The data show that this is true. There is a significant increase in the frequency of multipolar mitosis in high-grade dysplasia (5%) and oral cancer (7.2%). Similar findings have been reported for carcinoma in situ of the cervix (multipolar mitosis in ~ 10% of cells) and ductal carcinoma in situ of the breast (~16-17%) (Pihan et al., 2003). Truncation of the section during slide preparation could again result in a significant underestimation of this phenomenon. Another confounding factor is the low frequency of mitosis that is apparent in tissue sections. The above two reasons would explain why the percentage of abnormal mitotic cells was 0 in low-grade dysplasia.
VII.2. CURRENT ISSUES IN EVALUATING CENTROSOME SIZE ALTERATION

There is no consensus currently among researchers on how centrosome size (diameter) should be measured. In many studies, centrosomes are considered abnormal when their diameters are more than double the diameter of centrosomes in same-type normal cells or cells of other types that are present in the tissue (fibroblasts or lymphocytes). However, the ‘double the diameter’ norm was selected anecdotally without any scientific support. There is a lack of detailed information about distribution of centrosome diameters at different stages of carcinogenesis. Some studies have reported different mean diameters of centrosomes for different tissues: 0.46μm for normal oral cells, 1μm for pancreas tissue cells (Sato et al., 2001), 1μm for ovarian cells (Hsu et al., 2005) and 0.4μm for bladder cells (Jiang et al., 2003). Among the above studies, the mean centrosome diameters were decided by measuring a few cells only so the above data can’t be applied in general. There is a strong need for more information on size changes of centrosomes before setting any cutoff lines for oral carcinogenesis.

In this study, we first observe the distribution of centrosome diameters in 4 different histological groups (sees section VI.2 in Results). There is a trend towards an increased mean diameter of centrosomes with histological progression, present in both HGD and SCC in comparison to other histological groups. The diameter size of 0.64μm was selected as our cutoff line to determine abnormally enlarged centrosomes as 95% of the centrosomes measured in the 3,667 normal oral cells are equal to or smaller than this
measurement. By using this cutoff, we showed that there is an association between abnormally enlarged centrosomes and histology ($P < 0.001$). We believe that using the 95th percentile size of centrosomes in normal cells as a cutoff line is more appropriate than using double the mean centrosome size in normal cells for evaluating size abnormalities of centrosomes.

VII.3. AMPLIFIED $\gamma$-TUBULIN STAINED SIGNALS IN ORAL TISSUES

Cells with numerous, irregular and variously sized $\gamma$-tubulin stained signals scattered in the cytoplasm of cells (called amplified centrosomes) are frequent occurrences in the tumor samples examined in this thesis. This is not unexpected since similar findings have been reported for tumors at other sites including lung (Koutsami et al., 2006), pancrease (Sato et al., 1999), prostate (Pihan et al., 2001; Pihan et al., 2003), gall bladder (Kuo et al., 2000), oral SCC (Gustafson et al., 2000) and head and neck cancers (Carroll et al., 1999). What is striking, however, is the high frequency of such change in normal samples in this thesis. In other words, amplified centrosomes exist in normal tissue from individuals without any premalignant lesion or cancer. Very few studies report data on such samples as they restrict analyses to tumors. One study that examined pancreatic cancer reported similar diffuse staining centrosomes in some normal pancreatic tissues (Sato et al., 1999). Another study examined centrosomes in 18 oral cavity tumors, looking at both tumor areas and surgical margins. It found amplified centrosomes in both tumors (94% or cases) and margins (84%) (Gustafson et al., 2000). Another study
showed similar amplified centrosomes in hyperplastic areas adjacent to the cancer tissues (Koutsami et al., 2006).

In this study, we used a separate approach to determine whether centrioles are present in tissues with amplified centrosomes (γ-tubulin stained signals). This involves co-staining another section of each tissue with anti-γ tubulin antibody to stain the pericentriolar matrix areas of centrosomes and anti-α/β tubulin antibody to stain the centrioles. In normal tissues, almost all amplified centrosomes were acentriolar. The percentage of amplified acentriolar centrosomes drops slightly in low-grade dysplasia (99.5%), a little more in high-grade dysplasia (99%) and considerably more in cancer tissues (91%). This study is the first to investigate the presence or absence of centrioles in centrosomes from paraffin human tissues. To date, the presence of acentriolar centrosomes have been reported in few human cancer cell lines including breast cell line BT-549 and Hodgkin’s disease-derived cell line L428 using a similar procedure (Pihan et al., 1998). Another study also reported amplified acentriolar centrosomes (multiple centrosomes with missing centrioles) in CGL-2 cells, a hybrid (ESH5) of the HeLa variant, treated with 2μmol/L arsenite for 20 hours (Yih et al., 2006). Alternatively, the amplified γ-tubulin stained signals that lack centrioles might possibly be from γ-tubulin outside of the centrosomes.

Anti-γ tubulin antibody has been used widely as a centrosome marker in many different aspects of research during the last decade. However, γ-tubulin is only one of many centrosomal proteins and its existence does not ensure the presence of a complete
centrosome. Of interest, two types of γ-tubulin complexes have been identified in
centrosomes and in the cytoplasm. About 20% of γ-tubulin exists in centrosomes as γ-
tubulin ring complex (γTuRCs), which contain multiple copies of γ-tubulin small
complex (γTuSC) in association with GCP-4, GCP-5 and GCP-6 (Murphy et al., 2001). The other 80% of γ-tubulin
exists in the cytoplasm as γTuSCs, which contain only two molecules of γ-tubulin protein and one molecule of GCP-2 and GCP-3 (Murphy et al.,
1998; Fava et al., 1999). Under normal conditions, γTuSCs are too small to be detected
using fluorescence immunostaining (they have only about 1/55th of the γ-tubulin that is
present in centrosomal γTuRCs (Moudjou et al., 1996; Murphy et al., 1998; Fava et al.,
1999; Murphy et al., 2001). However, studies support the possibility that cytosolic γ-
tubulin protein could aggregate into multiple foci under certain conditions. For example,
in primary rat mesencephalic cells treated with rotenone, γ-tubulin proteins aggregated
into multiple varied sized spots around the nucleus (Diaz-Corrales et al., 2005). Hence,
amplified γ-tubulin stained signals could be simply aggregation of cytoplasmic γ-tubulin
under unknown mechanisms.

When γ-tubulin genes are overexpressed in monkey kidney COS cell line, numerous
aggregated γ-tubulin stained signals are scattered in the cytoplasm of cells while only two
γ-tubulin stained signals exist in control COS cells (Shu et al., 1995). Similar findings
are reported when γ-tubulin genes are overexpressed in mouse transformed Sertoli (TM4)
cells in vivo (Fleming et al., 2003). These data support the possibility that γ-tubulin
overexpression could be a possible explanation for the multiple scattered γ-tubulin

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stained signals in the cytoplasm of oral cells. However, there are no reports in studies of 
$\gamma$-tubulin gene amplification in human cancers.

False positive signals are also a possible explanation for this observed phenomenon. 
Several small studies were conducted to further explore this possibility. First, use of 
higher dilutions of primary and secondary antibodies did not affect the number of 
amplified $\gamma$-tubulin stained signals detected in these archival tissue samples. Second, we 
also used the same anti-$\gamma$ tubulin antibody on cells collected with exfoliated cell 
brushings of the oral cavity (fixed with formalin): amplified $\gamma$-tubulin stained signals 
were present in these specimens. Third, the anti-$\gamma$ tubulin antibody was applied to the 
tissue treated with different antigen retrieval (AR) methods, including different heated 
solutions, enzyme treatments and a combination of both of these approaches. Amplified 
centrosomes were still present. Based on the above tests, the likelihood of false positive 
signals has been minimized but not completely eliminated. There is still a chance that 
these stained signals are false positive signals to other proteins with binding structures 
similar to $\gamma$-tubulin.

As new proteins are identified as belonging to centrosomes, antibodies should become 
available that can be used to detect these different centrosomal proteins or different parts 
of $\gamma$-tubulin. With the help of these other antibodies, we might be able to determine the 
source of the amplified $\gamma$-tubulin stained signals. However, to date, most commercial 
antibodies can not detect centrosomes or $\gamma$-tubulin successfully in paraffin tissues.
VII.4. LIMITATIONS OF STUDY

Limitations of this study that could influence the accuracy of the results include sample size and technical issues related to fluorescence immunostaining. In this study, we collected 50 archived tissues and classified them into five different groups according to their histological diagnosis. To improve the accuracy of this study, we examined at least 400 cells in each tissue under each evaluation category. However, 4 to 13 samples in each group is not a sufficient sample size to represent the entire histological group, especially of early stage premalignant lesions with known heterogeneity.

Some factors related to fluorescence immunostaining techniques could influence the accuracy of results. These factors include fixation, antigen retrieval methods and antibody specificity. Fixation is the process used to preserve the cell morphology and proteins. During fixation, antigens could be damaged or destroyed through interactions between fixation buffers and proteins. Antigenicity also could be reduced due to inadequate fixation or inappropriate fixation solutions (Berod et al., 1981; Eldred et al., 1983; Shi et al., 1997). In this study, all samples were archived from hospitals. This means samples were biopsied at different time points by different individuals and processed by different technicians in the hospitals. This could lead to inconsistent fixation of samples and possibly variation in results between samples.

During the fixation process, the antigenicity of proteins will be modified and cause poor recognition of antibodies. In paraffin tissues, proper AR methods are an important step to recover the antigenicity of proteins. For example, one study reported that proper AR
treatment improved the p53-positivity up to 54% in paraffin tissues (Shi et al., 1997). Improper AR methods tend to reduce the positive rates of antibody staining and cause an underestimation (Cattoretti et al., 1993). Currently, there are no universal AR methods available for every antibody. Heat-induced AR methods provide better results in some conditions while enzyme induced AR methods provide better results in some other conditions (Wakamatsu et al., 1997; McNicol et al., 1998). To optimize the staining signals, in this study, we tested different AR methods prior to running the patient samples. However, there are many AR methods available and we may not have included the best AR method during our testing rounds. This might lead to underestimation of results.

Another important issue when using fluorescence immunostaining with paraffin tissues is antibody specificity. Poor antibody specificity causes false positive results due to cross-reactivity. This happens when two different proteins share identical epitopes or similar amino acid sequences. Both monoclonal and polyclonal antibodies could result in false-positive results although monoclonal antibodies have higher specificity and a lower chance of cross-reactivity. In this study, we tried to avoid false-positive results by selecting antibodies that have been well-known and widely used in centrosome studies to minimize the chance of cross reactivity due to differences in tissue types and techniques.
VII.5. FUTURE PLANS

This study provides the first indication of the potential value of examining centrosome abnormalities in OPLs for a possible key role in early oral carcinogenesis. We have reported alterations in centrosome size, shape and number that are present in both oral cancer and precancers. This suggests that such changes could be a driving force of chromosomal instability. If so, one would expect to see elevations in chromosome numbers associated with these centrosome alterations. This could be tested in the future using FISH analysis with chromosome-specific centromere probes in serial sections showing centrosome abnormalities. We also report in this study an association of two types of centrosome abnormalities (cluster formation and more than 2 centrosomes in one cell) with severity of histological change. Each of these anomalies could be potential risk predictors of cancer progression for early premalignant lesions. If so, one would expect to see an elevation in such changes in those early OPLs that later progressed to oral cancer. This possibility could be tested by examining dysplastic tissues of similar stage with known outcomes (some progressing, others not) to see if the centrosome anomalies were preferentially associated with progression. If any type of centrosome abnormalities could be used as a risk predictor, it would help in diagnosing high-risk OPLs at an earlier stage and improve the present poor 5-year survival rate.

Finally, there is also a need to understand more about the molecular mechanisms underlying centrosome abnormalities. There are a growing number of possible pathways in studies that could lead to centrosome abnormalities. Each pathway is related to
different protein alterations. The data presented in this thesis suggest that the oral histopathological model can help to identify those critical pathways and proteins associated with centrosome abnormalities and with chromosomal instability.

VII.6. CONCLUSION

This study was designed to investigate OPLs and cancer for centrosome abnormalities in formalin-fixed, paraffin-embedded tissues. To our knowledge, this is the first study examining centrosome abnormalities in OPLs. The results clearly show that both numerical and structural abnormalities of centrosomes exist in OPLs and cancers. The frequency of cells with abnormally enlarged centrosomes, cluster formation of centrosomes and presence of more than 2 centrosomes per cell were each associated with histological progression. These data support the concept that centrosome abnormalities are present in premalignant oral lesions and suggest that such change may play a role in inducing chromosomal change and in driving malignant transformation.
ABBREVIATIONS


Koutsami, M. K., P. K. Tsantoulis, M. Koulohoussa, K. Apostolopoulou, I. S. Pateras, Z.
Spartinou, A. Drougo, K. Evangelou, C. Kittas, J. Bartkova, J. Bartek and V. G.
Gorgoulis (2006). "Centrosome abnormalities are frequently observed in non-small-cell
lung cancer and are associated with aneuploidy and cyclin E overexpression." J Pathol

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Tanaka (2000). "Centrosome abnormalities in human carcinomas of the gallbladder and
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Lacey, K. R., P. K. Jackson and T. Stearns (1999). "Cyclin-dependent kinase control of

Lange, B. M. (2002). "Integration of the centrosome in cell cycle control, stress response and


villagers of oral precancerous lesions in Bilugyun: preliminary report." Community Dent

Kok (2005). "Univariate and multivariate analysis of prognostic significance of betel quid
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<td>% of cells with string-like structure</td>
<td>% of cells with total shape changes (cluster and string-like)</td>
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