Chapter 2

Literature review

2.1 Oral cancer

2.1.1 Definition

Oral cancer is a malignant neoplasm involving the lips, tongue, floor of mouth, gingival/alveolus (gum) and alveolus, palate and buccal mucosa (C00-C06). These defined oral cancer sites were based on the World Health Organization (WHO), International Classification of Diseases: tenth edition (ICD-10). The most common form of intra-oral malignancy is squamous cell carcinoma (Blot *et al*., 1992; Zakrzewska, 1999).

2.1.2 Epidemiology of oral cancer

Worldwide, oral cancer is ranked 11th most common malignancy (Sankaranarayanan, 2003). It accounted for about 274,000 cases with estimated mortality rate of about 127,000 deaths in the year 2002. Of these figures, two-thirds of which occurred in the developing countries (Parkin *et al*., 2005). In many parts of Asia, oral cancer continues to be a major health problem. The occurrences of oral cancer are dominated by countries in southern Asia, South-East Asia and Melanesia region (Johnson, 2003a). Oral cancer was reported as the most common site and accounts for about 40% of all cancers in India and Sri Lanka (Sankaranarayanan, 1990; Zakrzewska, 1999). However, in developed countries like United Kingdom (UK) and United States of America (USA), oral cancer only accounted for 1-2% of all cancers detected (Zakrzewska, 1999; Jemal *et*

al., 2007). Despite the low prevalence in the UK, more than 50% of oral cancer results in deaths (Zakrzewska, 1999).

In Malaysia, the second report of the National Cancer Registry (NCR) data revealed that a total of 21,464 cancer cases were diagnosed among Malaysians in Peninsular Malaysia in the year 2003 (Lim and Halimah, 2004). In this report, oral cancers were recorded separately into the lip, mouth and tongue cancers. Among the males, mouth and tongue cancers were ranked 19th and 17th respectively of all cancers in Malaysia. Meanwhile among the females, mouth and tongue cancers were ranked 16th and 21st respectively of all cancers. When both mouth and tongue cancers were taken together, cancers of the oral cavity would account for 2.5% of male cancers and 2% of female cancers making it the 12th commonest cancer among men and the 13th most common cancer among women.

NCR also reported that the age specific incidence rate (ASR) for both mouth and tongue cancers increased with age. With regards to ethnicity, Indians have a higher incidence of mouth cancers than the other races. In fact, the Indian females had the highest incidence of mouth cancers with an ASR of 16.5 per 100,000 of the population. Among Indian males, mouth and tongue cancers were ranked 6th (5.2%) and 9th (4.4%) respectively of all cancers. Meanwhile, mouth and tongue cancers were ranked 3rd (7.7%) and 9th (3.1%) respectively of all cancers among Indian females. When both mouth and tongue cancers were combined, they account for 9.6% of Indian male cancers making it the most common cancer among men and the 3rd most common cancer among Indian women (10.8%). NCR further described when comparing between the Indians and Malays, Indians were found to have 8.8 times the incidence of male mouth cancer, and 28.3 times the

incidence of female mouth cancer. With regards to tongue cancers, Indians had the highest incidence rate of tongue cancers which was 6.2 times the Malay male incidence and 11.3 times the Malay females.

Based on the Annual Report 1996 from the Ministry of Health, quoted by Zain and Ghazali (2001), lip and oral cancer were the third most common cancer deaths in government hospitals. In fact, this malignancy accounted for 7.1% of cancer deaths reported from the facilities of the Ministry of Health. Although prevalence of oral cancer is low in Malaysia at 0.04% (Zain *et al*., 1997), about 60% of oral cancer lesions have been found to occur among the Indian ethnic group who comprise only about 8% of the total Malaysian population (NCR, 2003).

2.1.3 Molecular epidemiology

Besides common epidemiological studies, quite a number of molecular epidemiology studies have also been conducted (London *et al*., 2000; Zhao *et al*., 2001; Seow *et al*., 2002; Wang *et al*., 2004). In a well-designed molecular epidemiological study, the risk associated with metabolic polymorphisms for oral cancer was assessed and so far have shown that the overall effect of common polymorphism is moderate in terms of relative risk (Nair and Bartsch, 2001). These type of studies also has enabled us to identify a number of carcinogenic hazards, and in some cases providing definitive etiologic data, besides furthering our understanding of individual genetic and acquired susceptibility to environmental carcinogens (Perera, 2003) and also development of oral cancer or oral carcinogenesis.

2.1.4 Oral carcinogenesis

Oral cancer is a multistep process in which multiple genetic events occur that alter the normal functions of oncogenes and tumor suppressor genes (Williams, 2000). The activation of oncogenes and the inactivation of tumor suppressor genes are the results of early genetic alterations that accompany phenotypic changes that occur in tumor progression (Kupferman and Myers, 2006). The consequences of this genetic damage are cell dysregulation with disruption in cell signaling, cell growth cycle, and/or mechanisms to repair cell damage or eliminate dysfunctional cells. It is the accumulation of such genetic changes, often over a period of time that becomes autonomous and with invasive mechanisms developed would lead to a carcinoma (Scully *et al*., 2000). Figure 2.1 illustrates these events.

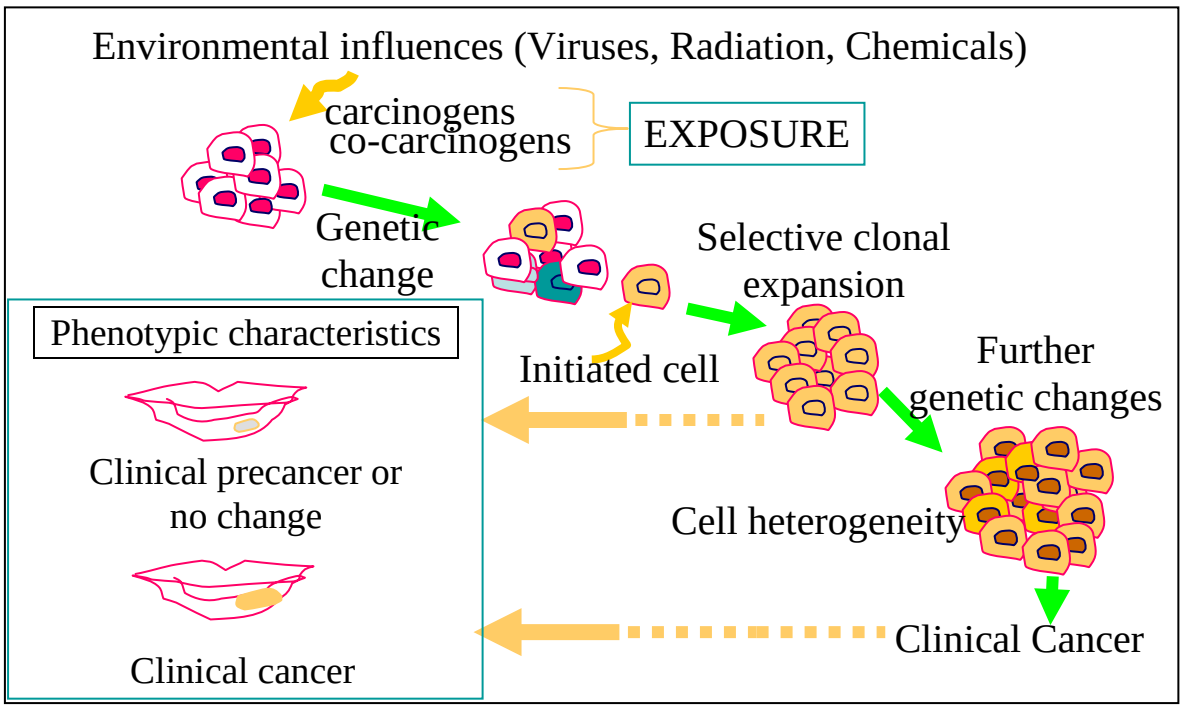

Figure 2.1: Continuum of events between exposure and disease (cancer).

(Source: Illustration based on concepts of molecular epidemiologic events from A Conceptual and Historical Framework for Molecular Epidemiology by Schulte, 1993).

Cancers are often perceived as the outcome of a complex biological process. The stages of carcinogenesis consists of initiation which include the DNA damages to the cells or tissues as a result of exposure to carcinogens, followed by division of the exposed cells, such that their growth potential is changed irreversibly, and lastly progression indicating multiple rounds of cell replication mediating the gradual transition of an initiated cell towards autonomous cancerous growth (Figure 2.1). Metastasis represents the ultimate spread of malignant cells resulting in multiple tumor sites (Stewart, 2003).

2.1.4.1 Oncogenes

Oncogenes are genes whose protein products have been found to be important for normal cell growth signaling and differentiation. However, over-expression or mutation of these genes leads to unchecked cell growth and tumorigenesis (Das and Nagpal, 2002). Mechanisms of activation of these cellular oncogenes include point mutations and DNA rearrangements (Wong *et al*., 1996). In oral cancer, some of the oncogenes involve are EGFR, STAT3 and K-RAS (Kupferman and Myers, 2006).

2.1.4.2 Tumor suppressor genes

Tumor suppressor genes, on the other hand, encode proteins that prevent normal cellular processes from going awry and are likely to be involved in regulating cell growth or differentiation, cell-cycle control, cell-cell adhesion, apoptosis and maintenance of genomic integrity (Fearon, 2002). In general, cells require only a single functioning copy of the tumor suppressor gene to maintain normal cellular homeostasis. However, when a cell undergoes loss of both alleles of a tumor suppressor gene locus, through deletion,

mutation, or epigenetic silencing, the loss of cell growth control become evident (Das and Nagpal, 2002). This process has since been observed for numerous tumor suppressor genes, including pRB, p16, p53, APC and BRCA (Kupferman and Myers, 2006).

2.2 Risk factors

In general, oral cancer development is influenced by several risk factors either environmental or genetic associated. Some of the environmental risk factors include tobacco smoking, alcohol drinking, betel-quid chewing, radiation and diet. Meanwhile some of the genetic associated risk factors are viruses and genetic predisposition. Of these, major risk factors such as tobacco smoking and excessive alcohol consumption are very well established (Johnson, 2003b). From the estimates of relative risk for tobacco habit and alcohol abuse, it has been estimated that 75% of all oral cancers could be prevented (Walker *et al*., 2003). The primary cause of high incidence in Asians is the widespread habit of chewing betel-quid (Johnson, 2003b). In a separate study, Winn *et al*., (1991) found that the regular use of mouthwash with high alcohol content may also contribute to oral cancer risk. The risk was more apparent when the alcohol content of the mouthwash exceeded 25 percent.

In the Malaysian context, a study done by Muttalib *et al*., (2002) revealed that a total of 44.5% of 6,781 subjects professed to one or more of the three 'high-risk' habits (namely tobacco smoking, alcohol drinking and betel-quid chewing). It was further discovered in his study that more than 22% of the locals studied practiced betel-quid chewing and 21.8% smoked. In fact, a higher proportion of females chewed betel-quid but

higher proportions of males smoked and consumed alcohol (Muttalib *et al*., 2002). In Taiwan, the risk factors for oral cancer were found to be very similar with the Malaysian population. Majority of the Taiwanese population also practiced the risk habits of cigarette smoking, alcohol drinking and betel-quid chewing (Hung *et al*., 1997).

2.2.1 Tobacco smoking

Tobacco smoking is the main known cause of human cancer-related death worldwide. In most developed countries, tobacco accounts for as much as 30% of all malignant tumors (Boffetta, 2003b). Tobacco smoking is the strongest risk factor for lung (Boyle and Maisonneuve, 1995) and oral cancer (Nair and Bartsch, 2001; Geisler and Olshan, 2001). Smoking of cigarette or bidi among the Indians has also shown to increase oral cancer risk. There is also a strong dose-response relationship found between the number of cigarettes smoked and the development of oral cancer (Vecchia *et al*., 1997; Geisler and Olshan, 2001; Reichart, 2001; Boffetta, 2003b).

Generally, the content of a cigarette consists of compounds such as tar, nicotine and nitrosamines which vary greatly, depending on species, curing additives and method of combustion (Johnson, 2001). In Malaysia, types of tobacco smoking includes commercial brand cigarettes which are the most common form, ‘bidi’ mainly used by Indians, hand-made paper-rolled cigarette (raw tobacco rolled in special paper prior to smoking), ‘rokok daun’ (raw tobacco rolled in temburna leaves prior to smoking) and kretek (an Indonesian imported type of cigarette containing spices such as cloves in addition to tobacco) (Zain and Ghazali, 2001).

Tobacco smoke contains a great number of chemical carcinogens (Boffetta, 2003b). The most important carcinogens and found abundantly in tobacco smoke are the polycyclic aromatic hydrocarbon benzo(α)pyrene and the tobacco specific nitrosamines (TSNs) namely 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN). These TSNs can induce specific mutations, especially guanidine-to-thymidine transversions, which interfere with DNA replication (Scully *et al*., 2000; Johnson, 2001; Walker *et al*., 2003). The metabolism of these carcinogens involves oxygenation by P450 enzymes in cytochromes and conjugation by glutathione s-transferase (GST). Some of these enzymes are polymorphic and strongly influence individual biological responses to carcinogens through their role in adduct formation (Scully *et al*., 2000). Therefore, genetic polymorphisms in the genes coding for these enzymes (for example GST) that result in increased carcinogen exposure may be the reason for individual susceptibility to cancer (Park *et al*., 1997; Johnson, 2001; Hashibe *et al*., 2003).

To reflect on the Malaysian population, findings from the Second National Health and Morbidity Survey 1996 revealed that the prevalence of current smokers aged 18 and above was 24.8%. This survey also showed that the highest prevalence of current smokers was among the Malays and the male gender of indigenous people of Sabah and Sarawak who had low level of education and household income (Haniza *et al*., 1999).

2.2.2 Alcohol drinking

Alcohol consumption has been strongly implicated as an independent risk factor in the development of oral cancer (Nair and Bartsch, 2001; Geisler and Olshan, 2001). Taken

together, alcohol consumption with tobacco smoking has been shown to act synergistically in increasing oral cancer risk (Blot, 1992; Reichart, 2001; Geisler and Olshan, 2001; Das and Nagpal, 2002; Kupferman and Myers, 2006). Johnson (2001) further reported that a poor diet together with the effects of tobacco smoking and heavy alcohol consumption had constituted over 90% of cases of head and neck cancer. Besides, these patients who diagnosed with oral cancer that were often exposed to tobacco and alcohol may also pose for higher risks of recurrences and second primary lesions (Kupferman and Myers, 2006).

All forms of alcoholic drink are dangerous if heavily consumed. In fact, alcohol may lead to nutritional deficiencies which could increase the susceptibility to carcinogens and also lead to immune suppression (Das and Nagpal, 2002). Among the commonly consumed alcoholic beverages locally in Malaysia are beer and stout. Special home-brands such as toddy and samsu are used by the Indians and domestically manufactured rice alcohols are used by the indigenous people of Sarawak (Zain, 1999).

The mechanism of alcohol causing cancer has been difficult to establish. However, there have been studies suggesting the possible pathways of alcohol inducing carcinogenesis (Vecchia *et al*., 1997; Du *et al*., 2000; Figuero-Ruiz *et al*., 2004). Firstly, alcohol being a solvent by nature has its ability to dry the surface of the oral mucosa, thus increasing the permeability which leads to easier penetration of carcinogens into the oral mucosa (Das and Nagpal, 2002). For instance, concentrations of ethanol of 25% and above significantly increased the permeability of porcine oral mucosa to NNN (Du *et al*., 2000). Other than ethanol, there are some carcinogenic chemicals present in alcoholic beverages such as N-nitroso compounds, mycotoxins, urethane, inorganic arsenic and others (Blot, 1992).

The major metabolite of alcohol is acetaldehyde whose transformation is mainly carried out by the enzyme alcohol dehydrogenase (ADH). Acetaldehyde is then oxidized to acetate by means of aldehyde dehydrogenase (ALDH). Acetaldehyde causes DNA damage in cultured mammalian cells. It interferes with DNA synthesis and repair. Acetaldehyde inhibits the enzyme 6-methylguanitransferase which is responsible for repairing injuries caused by alkylating agents. With all the above ill-effects of acetaldehyde which initiates or promotes tumor formation, increase in acetaldehyde accumulation in the body either due to increase in its production or due to decrease in its elimination, is considered harmful (Figuero-Ruiz *et al*., 2004). Accumulation of acetaldehyde can occur due to increased activity of ADH enzyme which is present in oral microflora and in the oral mucosa. Poor oral hygiene with increasing microbial flora can increase acetaldehyde accumulation. ADH type-3 genotypes cause rapid oxidation of alcohol to acetaldehyde and these individuals are more predisposed to oral cancer. Alternately, reduction in ALDH enzyme can also lead to accumulation of acetaldehyde (Figuero-Ruiz *et al*., 2004). Genetic polymorphisms have been reported in these two enzymes ADH and ALDH, which have been related to increased risk of alcohol-related cancers.

2.2.3 Betel-quid chewing

Several epidemiological studies have shown an association between the habit of betel-quid chewing and oral cancer and various precancerous lesions. The association has been consistent across many countries such as in India, Philippines, Malaysia, Bangladesh, Cambodia and Thailand (Saub, 2001). Among the Indians, chewing betel-quid with tobacco is the most widespread and has been demonstrated as a major risk factor for oral cancer (Gupta and Nandakumar, 1999).

Betel-quid chewing with different ingredients is the most common habit in Southeast Asia especially in the Indian subcontinent. Betel-quid (also referred to as pan or paan) usually consists of betel-leaf (leaf of *Piper betel* vine) that is wrapped around a mixture of cured or sun-dried areca nut (seed of *Areca catechu*), slaked lime (boiled from seashells) and tobacco (Kumar and Zain, 2004). The slaked lime lowers the pH and accelerates the release of an alkaloid from both the tobacco and areca nut, which produces a feeling of euphoria and well-being in the chewer (Johnson, 2001; Neville and Day, 2002). Considerable research has been focused recently on the carcinogenic, mutagenic and genotoxic potential of betel-quid ingredients, especially tobacco and areca nut.

In Malaysia, the single habit of chewing betel-quid was most popular among the Indian females (Ramanathan and Lakshimi, 1976). In a study conducted by Muttalib *et al*., (2002), it was reported that more than 22% of the population still practiced betel-quid chewing although it was now more confined to certain populations including Indians working in remote plantations, the indigenous people of Sabah and Sarawak and some elderly Malay folks living in rural villages (Zain and Ghazali, 2001). Most Chinese do not indulge in betel-quid chewing habit. Even after three decades, she reported that the higher proportion of females who chewed betel-quid remain the same while higher proportions of males tended to smoke and consume alcohol (Muttalib *et al*., 2002).

Betel-quid chewing produces reactive oxygen species (ROS) that have multiple detrimental effects upon the oral mucosa. The ROS can be directly involved in the tumor initiation process, by inducing genotoxicity and gene mutation or by attacking the salivary proteins and oral mucosa. This will eventually lead to structural changes in the oral mucosa

that may facilitate the penetration by other betel-quid ingredients and environmental toxicants (Walker *et al*., 2003).

2.2.4 Diet

Apart from tobacco smoking, alcohol consumption and betel-quid chewing habits, several dietary factors have been related to oral cancer risk (Fioretti *et al*., 1999). Antioxidants which are contained in fruits and vegetables seem to have a preventive effect (Reichart, 2001; Boeing *et al*., 2006). Generally, antioxidants like vitamin A, C, E and β-carotene scavenge potentially free radicals from damaged cells and are obtained in red, yellow and green fruits and vegetables (Reichart, 2001; Warnakulasuriya, 2002).

Dietary factors seem to be important in the prevention of oral cancer as has been shown in a number of recent studies. A study by Prasad *et al*., (1995) suggested that the poor dietary intake of vegetables and fruits coupled with low estimated intake of beta carotene, thiamin, riboflavin, folate, vitamin C, iron, copper and high saturated fat intake (Fioretti *et al*., 1999) may modify the oral cancer risk potential. The combined effects of micro nutrients appear to be protective in countering the adverse effects of exogenous exposures to tobacco (Prasad *et al*., 1995). Donaldson (2004) in his study highlighted that abundant portions of fruits and vegetables lowered cancer risk. Allium and cruciferous vegetables are especially beneficial in cancer prevention, with broccoli sprouts being the densest source of sulforophane. Protective elements in a cancer prevention diet also include selenium, folic acid, vitamin B12, vitamin D, chlorophyll and antioxidants such as the carotenoids (beta carotene, lycopene, lutein).

2.2.4.1 Functional foods

Food provides nutrients in infinite combinations and with unlimited variety. Functional foods can be defined as foods that provide health benefits beyond basic nutrition. Many fruits and vegetables fit into this category. For instance, broccoli and other cruciferous vegetables have been associated with a decreased cancer risk in epidemiological studies. Further research has shown that these vegetables contain a number of phytochemicals that have anticancer properties (Grosvenor and Smolin, 2002).

2.2.4.2 Phytochemicals

Phytochemicals are health-promoting compounds found in plant foods. These health-promoting properties have been recognized because of epidemiological observations that identified relationships between diets high in certain plant foods and a reduction in chronic disease such as cancer (Grosvenor and Smolin, 2002). Further evaluation of these foods has led researchers to specific phytochemicals that may be responsible for health benefits. Foods such as cruciferous vegetables and garlic have been found to be excellent sources of these health-promoting compounds. The phytochemicals found in these foods include indoles, isothiocyanates, dithiolthione and allium compounds (Craig, 1997).

Most phytochemicals are found in more than one type of plant food and many have multiple actions within the body. Sulfides and isothiocyanates stimulate the activity of enzymes that help deactivate carcinogens (Grosvenor and Smolin, 2002). For the purpose of this study, phytochemical such as isothiocyanate and their food sources and mechanism of action are discussed here.

2.2.5 Human papilloma virus (HPV)

HPV may play a role in the etiology of oral cancer (Warnakulasuriya, 2002). The exact role of HPV in oral cancer etiology still remains unclear. Williams (2000) in his study reported that HPV was found in 33-50% of oral cancer and mainly consist of HPV-16 and HPV-18. In another study, HPV-16 and HPV-18, which are well known for their oncogenic potential in uterine cervix cancer, are present up to 80% in oral cancer (Reichart, 2001).

The major evidence of the role of HPV in cancer development is that their genes and gene products are capable of disturbing the cell cycle machinery. HPV encodes two major oncoproteins namely, E6 and E7. The E6 and E7 proteins have been shown to bind and destroy p53 and pRb tumor suppressor genes respectively, thereby disrupting the cell cycle with loss of control on DNA replication, DNA repair and apoptosis (Warnakulasuriya, 2002).

2.2.6 Genetic susceptibility

Several important risk factors that may potentially increase oral cancer risk such as the use of tobacco, alcohol and betel-quid are all well recognized. However, despite the importance of these casual habits, relatively only a few people who practiced these habits actually develop cancer. Conversely, there are patients who develop oral cancer in the absence of such habits or other identifiable lifestyle or environmental etiologic factors. Therefore, these observations suggest that the importance of inherent genetic factors may play a role in the development of oral cancer (Scully *et al*., 2000).

Some patients appear susceptible to cancer because of an inherited trait which affects their ability or inability to metabolize carcinogens or procarcinogens (Scully *et al*., 2000; Kumar and Zain, 2004). Individual susceptibility to cancer is likely to be modified by the genotype for enzymes involved in the activation or detoxification of carcinogens in the human body which was constantly exposed to the environmental risk factors. Differences in metabolic capacity due to inherent genetic differences in Phase I (cytochrome P450) and phase II (GST drug metabolizing and detoxifying enzymes) enzymes indicate that genetic constitution may predispose an individual towards cancer (Johnson, 2001; Das and Nagpal, 2002).

For instance, in a comparative study of African American and white American patients with oral cancer in the United States, *GSTM1* null genotype was found to carry a significantly high oral cancer risk among African Americans but not among whites (Park *et al*., 2000). The lack of significant associations between *GSTM1* genotype and oral cancer risk in Caucasians was further confirmed by a study on *GSTM1*, *GSTT1* and *CYP1A1* in 185 Netherlands head and neck cancer subjects unmatched to 207 control individuals (Oude Ophuis *et al*., 1998).

Other than *GSTs* polymorphisms, there are genetic susceptibilities reported among Japanese subjects which include polymorphism of N-acetyltransferase 1 and a finding that *NAT*10* allele had a significantly high relative risk of 5.9 for non-smokers and 3.1 for smokers (Katoh *et al*., 1998). Genetic polymorphism of aldehyde dehydrogenase-2, which eliminates acetaldehyde generated during alcohol metabolism was examined in 237 Japanese alcoholics (Yokoyama *et al*., 1998), of whom 16 had SCC of oral cavity. Fifty percent who had SCC of oral cavity had the mutant *ALDH2*2* allele, and the blood

concentration of acetaldehyde in the affected 16 subjects was 11 times greater than in the homozygotes, and in this subset, significantly more multiple primary tumors were found.

Determination of the nature of these genetic factors would have enormous benefit, not only to at risk family members, who would thus take particular care to avoid other risks, but in unraveling the molecular mechanisms of oral carcinogenesis, opening the way to better prevention and treatment (Johnson, 2003b).

2.3 Dietary isothiocyanates (ITCs)

ITCs are a family of compounds derived almost exclusively from plants and found abundantly in vegetables (Zhang, 2004). ITCs are largely responsible for the characteristic hot, pungent flavors of salad vegetables such as radish, cress, mustard leaves and watercress, and contribute to the flavor of cooked cruciferous vegetables (IARC, 2004).

All ITCs are characterized by the presence of an –N=C=S group, whose central carbon often is highly electrophilic. The biological activities of ITCs, perhaps their toxic effects, may be primarily mediated through the reaction of this carbon atom with cellular nucleophilic targets. It is believed that the side chains of ITCs may play secondary roles, for example, affecting the electrophilicity of the –N=C=S group, altering the steric hindrance to the reactive carbon atom, and controlling the lipophilicity of the molecule (Kolm *et al*., 1995; Meyer *et al*., 1995; Zhang *et al*., 1995). Some of the structures of ITCs found commonly in consumed cruciferous vegetables are as in Figure 2.2.

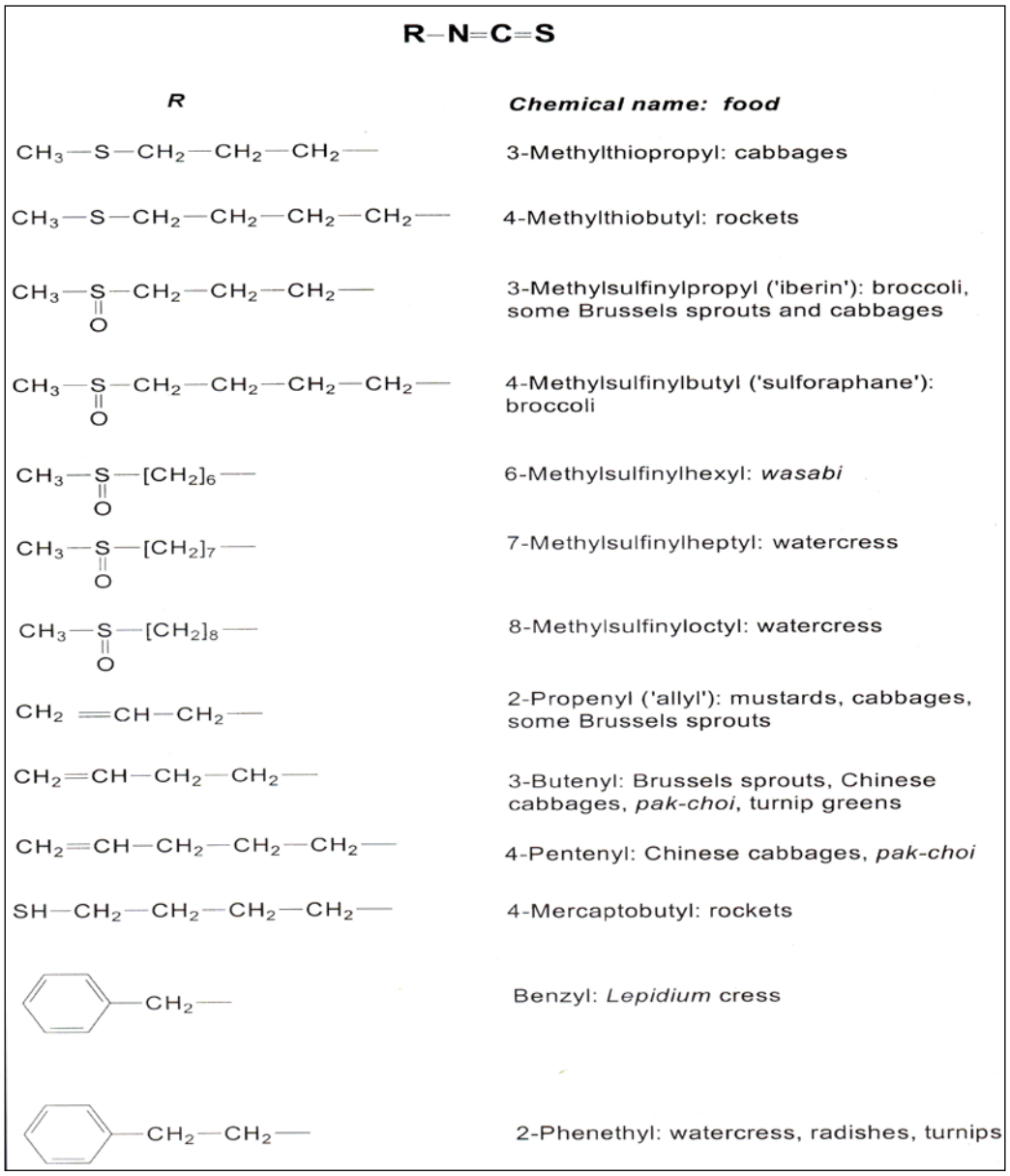

Figure 2.2: Structures of isothiocyanates and side-chain structures (R) found commonly in eaten cruciferous vegetables.

(Source: IARC Handbooks of Cancer Prevention, Cruciferous Vegetables, Isothiocyanates and Indoles, Vol. 9, WHO, IARC Press, 2004)

ITCs are synthesized and stored in plants as relatively stable precursors, known as glucosinolates (Fahey *et al.*, 1997). When the plant cells are injured or damaged, by microbial attack, mechanical food processing or chewing, glucosinolates are released and

converted to ITCs by the action of enzyme myrosinase (Shapiro *et al*., 1998). Myrosinase is an enzyme that coexists with but is physically segregated from glucosinolates in normal plants cells (Zhang, 2004). Myrosinase catalyses the following reaction (Figure 2.3):

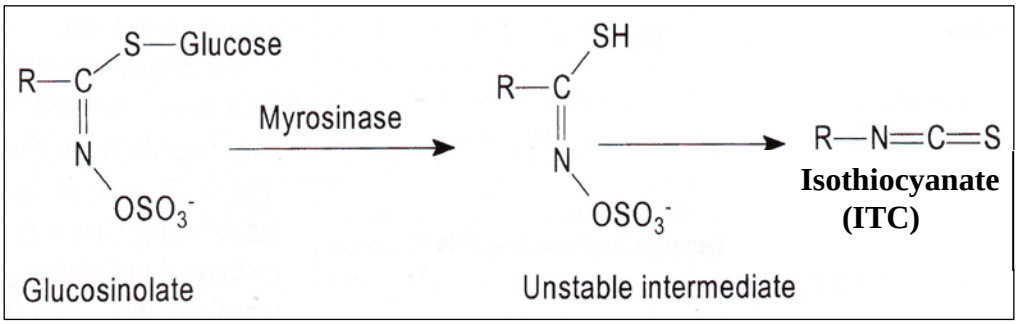

Figure 2.3: Hydrolysis of glucosinolates to isothiocyanates.

(Source: IARC Handbooks of Cancer Prevention, Cruciferous Vegetables, Isothiocyanates and Indoles, Vol. 9, WHO, IARC Press, 2004)

During cooking of cruciferous vegetables (brassica), the glucosinolate-myrosinase system may be modified as a result of inactivation of plant myrosinase, loss of enzymic cofactors such as epithiospecifier protein, thermal breakdown and/or leaching of glucosinolates and their metabolites or volatilization of metabolites (Dekker *et al*., 2000). Cooking brassica affects the site of release of breakdown products of glucosinolates, which is the upper gastrointestinal tract following consumption of raw brassica containing active plant myrosinase. After consumption of cooked brassica devoid of plant myrosinase glucosinolates are hydrolysed in the colon under the action of the resident microflora (Rungapamestry *et al*., 2007). The digestive fate of glucosinolates may be further influenced by the extent of cell rupture during ingestion, gastrointestinal transit time, meal composition, individual genotype and differences in colonic microflora (Stahl *et al*., 2002).

Thus, it is also important to determine the levels of ITCs in cooked vegetables that have been prepared in the usual ways by the studied populations (Jiao *et al*., 1998).

The metabolism of ITCs is governed by the –N=C=S groups. ITCs are metabolized *in vivo* principally by the mercapturic acid pathway: an initial conjugation through the –N=C=S group with glutathione (GSH), which takes place spontaneously but is further promoted by GST (Kolm *et al*., 1995; Meyer *et al*., 1995; Zhang *et al*., 1995), giving rise to the corresponding conjugates. The GSH conjugates then undergo further enzymatic modifications (modifications of the GSH portion) to form sequentially the cysteinylglycine-, cysteine- and N-acetylcysteine –conjugates, which are excreted in urine.

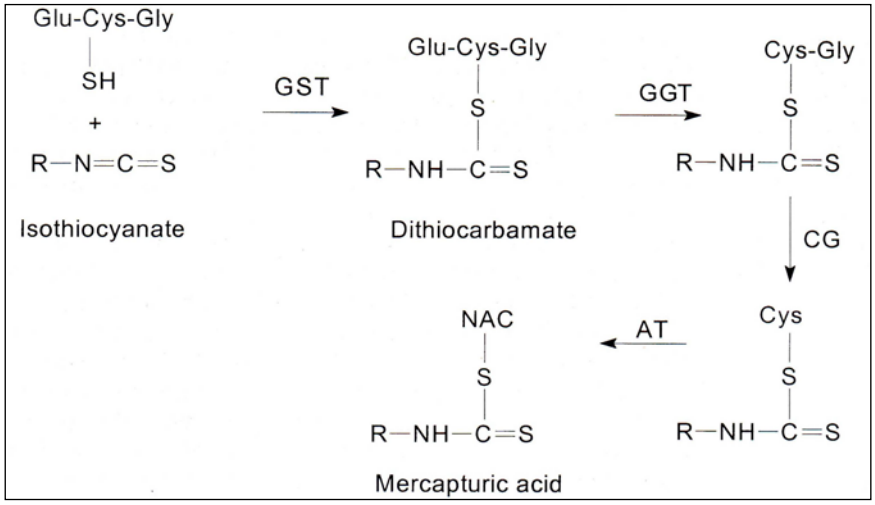

Figure 2.4: Isothiocyanates are conjugated to glutathione by glutathione s-transferase (GST), metabolized sequentially by γ-glutamyl transpeptidase (GGT), cysteinylglycinase (CG) and N-acetyltransferase (AT) to form, ultimately, mercapturic acid, NAC, N-acetylcysteine.

(Source: IARC Handbooks of Cancer Prevention, Cruciferous Vegetables, Isothiocyanates and Indoles, Vol. 9, WHO, IARC Press, 2004)

2.3.1 Sources

Cruciferous vegetables are the principle dietary source of ITCs, but the types of crucifers frequently consumed by humans are limited. Below are some examples of popular crucifers that are particularly rich in certain ITCs include broccoli, cauliflower, bak choy, cabbage, Brussels sprouts, kai lan, watercress and choy sum (Zhang, 2004).

Figure 2.5: Broccoli

(Source: http://en.wikipedia.org/wiki/Broccoli)

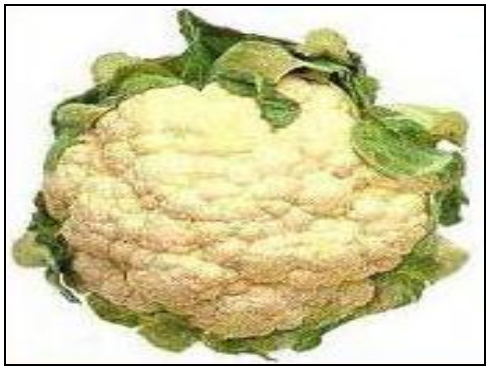

Figure 2.6: Cauliflower

(Source: http://en.wikipedia.org/wiki/Cauliflower)

Figure 2.7: Bak choy

(Source: http://en.wikipedia.org/wiki/Bok_choy)

Figure 2.8: Cabbage

(Source: http://en.wikipedia.org/wiki/Cabbage)

Figure 2.9: Brussels sprouts

(Source: http://en.wikipedia.org/wiki/Brussels_sprout)

Figure 2.10: Kai lan

(Source: http://en.wikipedia.org/wiki/Kai-lan)

Figure 2.11: Watercress

(Source: http://en.wikipedia.org/wiki/Watercress)

Figure 2.12: Choy sum

(Source: http://en.wikipedia.org/wiki/Choy_sum)

2.3.2 Estimated dietary ITC intake

Many efforts had been made to obtain estimates of dietary intake of ITC. So far, only Jiao *et al*., (1998) in Singapore and Shapiro *et al*., (1998) in the USA had reported the total ITC concentration found in several cruciferous vegetables. The estimates however,

are only approximate as the concentrations of ITC vary considerably depending on the growing conditions, the cultivars, storage and preparations, as considerable amounts of glucosinolates can be lost during storage and processing (IARC, 2004).

Table 2.1: Cruciferous vegetables and their total ITC contents

Vegetables	ITC contents, mean and range (μmol/100g wet weight)
Broccoli (*Brassica oleracea* var. *italica*)	38.6 (10.1 – 62.0)
Cabbage (*Brassica oleracea* var. *capitata*)	27.5 (11.9 – 62.7)
Cauliflower (*Brassica oleracea* var. *botrytis*)	11.6 (2.7 – 24.0)
Choy sum (*Brassica chinensis* var. *parachinensis*)	11.1 (3.5 – 23.4)
Kai lan (*Brassica* var. *alboglabra*)	15.4 (3.1 – 35.9)
Watercress (*Nasturtium officinale*)	81.3 (17.1 – 144.6)

(Source: Jiao *et al*., 1998)

To date, the most important known biological activity of ITCs is their ability to inhibit cancer development. Many ITCs are potent cancer chemoprotective agents in animal systems (Zhang, 2001). A distinctive feature of these cruciferous vegetables is their relatively high content of glucosinolates, which are converted *in vivo* to ITCs, indoles and nitriles by the enzyme myrosinase (Steinkellner *et al*., 2001).

2.4 Genetic polymorphisms

Genetic polymorphisms are a form of mutation which occurs in more than 1% of a population. These polymorphisms also refer to the simultaneous occurrence in the population of genomes which gives rise to allelic variations (as seen either in alleles producing different phenotypes). Genetic polymorphisms are associated with a number of genes which code for enzymes involved in the metabolic activation or detoxification of carcinogens (Park *et al*., 2000). For example, polymorphisms of *GSTM1* and *GSTT1* genes involve deletion of alleles while the polymorphism of *GSTP1* involves the amino acid substitution of isoleucine with valine.

2.4.1 Glutathione s-transferases (GSTs)

Glutathione S-transferase (GST) is a family of genes which are involved in the metabolism of many xenobiotics, including an array of environmental carcinogens. Human *GSTs* comprise several subfamilies of isoenzymes: principally *GSTM1*, *GSTT1* and *GSTP1*. These enzymes are involved in the detoxification of the activated metabolites of carcinogens (Hung *et al*., 1997; Scully *et al*., 2000). *GSTM1* and *GSTT1* genes can be indicated by non-null (presence) or null (absence) genotypes. As for *GSTP1* gene, ile/ile or wild-type genotype referred to high activity of the enzyme while the ile/val or val/val genotypes (polymorphism) indicate low activity of the enzyme. Deletions in the *GSTM1* and *GSTT1* genes can produce the null genotypes, which lead to absence of activity of these enzymes; similarly reduced activity of *GSTP1* has been attributed to the low activity of ile/val and val/val alleles.

GSTs also are dimeric proteins that catalyze conjugation reactions between glutathione and tobacco smoke substrates, such as aromatic heterocyclic radicals and epoxides. Conjugation facilitates excretion and thus constitutes a detoxification step (Geisler and Olshan, 2001). This class of enzymes is therefore important for maintaining cellular genomic integrity and, as a result, may play an important role in cancer susceptibility.

The *GSTM1* gene locus has been mapped on chromosome 1p13.3. This gene was found to be related in susceptibility to squamous cell carcinoma of the head and neck (Geisler and Olshan, 2001). The GSTM1 enzyme play an important role in the detoxification of polycyclic aromatic hydrocarbons (PAHs) including the strong tobacco smoke carcinogen and benzo(α)pyrene (Park *et al*., 2000). In the detoxification step, GSTM1 enzymes help to catalyze the conjugation of glutathione to carcinogenic diol-epoxide, derivatives from PAHs which will be excreted (Mannervik *et al*., 1988). Individuals who have null genotype for the *GSTM1* gene will have no GSTM1 enzyme activity. Hence, it has been suggested that the lack of this enzyme may potentially increase cancer susceptibility because of a decreased inability to detoxify carcinogens such as benzo(a)pyrene-7,8-diol epoxide, the activated form of benzo(α)pyrene (Hashibe *et al*., 2003).

As for the *GSTT1*, its gene locus exists on chromosome 22q11.2. This GSTT1 enzyme has high activity towards epoxy and peroxide compounds (Meyer *et al*., 1991) and metabolizes alkyl halides and lipid peroxides (Scully *et al*., 2000). GSTT1 enzyme is important in the detoxification of naturally occurring monohalomethanes, as well as the industrial compounds dichloromethane and ethylene oxide (Pemble *et al*., 1994). Because

these agents are widely used as methylating agents, fumigants, pesticides and solvents, polymorphisms involved in their metabolism may be of importance in the etiology of aero-digestive cancers. Individuals who have null GSTT1 locus have no enzymatic functional activity (Geisler and Olshan, 2001). Lacking a functional GSTT1 enzyme reportedly cannot conjugate monohalomethanes found in tobacco smoke and may have greater susceptibility to chromosomal damage via sister chromatid exchanges. Accumulation of these carcinogens in the body may increase the cancer risk.

The *GSTP1* gene is located on chromosome 11q13 (Rossini *et al*., 2002; Cote *et al*., 2005). *GSTP1* is one of a family of GST isozymes which catalyze the addition of glutathione to a broad spectrum of chemical compounds including ethacrynic acid and acrolein, as well as potent carcinogens such as epoxides of PAHs (Ketterer *et al*., 1992). *GSTP1* is the most abundant of the GST enzymes and has been shown to be ubiquitously expressed in most human tissues including the oral cavity (Sarkar *et al*., 1997). One polymorphism of *GSTP1* is caused by a single base pair substitution, where (A) adenine is replaced by (G) guanine, leading to an amino acid substitution in which isoleucine (I105) is replaced by valine (V105) (Miller *et al*., 2003). The polymorphism of *GSTP1* genotype will generally lower activity towards PAH diol epoxides, and thus, has been predicted to have lower detoxification potential and greater risk for cancer.

2.4.1.2 Distribution of GSTs

The distribution of polymorphisms related to *GSTs* has been reported in different populations, mainly in the Asian region. Table 2.2 summarizes the distribution of GST genotypes among the population. Except for the study in Korea and Singapore which only

consists of males and different races respectively, the studies for other *GSTs* polymorphic genotypes from various population were being gender and ethnic independent.

Studies from Korea and Southern China have showed that the distribution of *GST* were similar between these two populations. This similarity was clearly seen in *GSTM1* null, *GSTP1* ile/ile (wild-type), ile/val and val/val genotypes where the distribution among the Korean were 53.8%, 68.4%, 29.1% and 2.5% (Cho *et al*., 2005), respectively as compared to the Chinese from China with 54.3% (*GSTM1* null), 60.7% (*GSTP1* ile/ile – wild-type), 35.2% (*GSTP1* ile/val) and 4.1% (val/val) (Zhong *et al*., 2006).

Two studies from South India also found consistent distribution of GST ranging from 22.4% – 30.4% and 16.8% - 17.6% for *GSTM1* null and *GSTT1* null genotype, respectively (Naveen *et al*., 2004; V *et al*., 2006). The *GSTT1* null distribution among the Indians was comparable between two countries namely India and Singapore. The similarity of *GSTT1* null genotype was noted with 16.0% and 16.8% among the Indians in Singapore (Lee *et al*., 1995) and India, respectively. The consistency was also obvious for both *GSTM1* and *GSTT1* combined genotype, the distribution among the Indians in these countries recorded 5.0% (Singapore) and 4.6% (India).

Despite the similarity among the Asian population, the distribution of the GST was found to differ between the Caucasian and the Asian group. This was especially noted in the distribution of *GSTM1* null, *GSTT1* null, *GSTP1* (ile/ile – wild-type) and *GSTP1* (val/val) genotypes. The distribution of *GSTM1* null and *GSTP1* (val/val) genotypes tended to be higher among the Caucasian than the Asian population. Conversely, the distribution of *GSTT1* null and *GSTP1* (ile/ile – wild-type) genotypes was more obvious among the

Asian than the Caucasian population. The percentage of mean distribution of *GSTM1* null and *GSTP1* (val/val) genotypes among the Caucasian were 49.5% and 12.4%, respectively as compared to the Asian population of 38.2% (*GSTM1* null) and 3.2% (*GSTP1* val/val). As for the *GSTT1* null and the *GSTP1* (ile/ile – wild-type) genotypes, the mean distribution among the Asian population were 35.4% and 62.5%, respectively as compared to the Caucasian of 21.6% (*GSTT1* null) and 46.4% (*GSTP1* ile/ile – wild-type).

Among the Brazilian (Caucasian), 42.1% of the individuals had *GSTM1* null genotype, whereas 25.4% had the *GSTT1* null genotype. The genotypic distribution of *GSTP1* was 49.7% (ile/ile – wild type), 38.1% (ile/val) and 12.2% (val/val). Individuals with both *GSTM1* and *GSTT1* null genotype was 9.8% (Rossini *et al*., 2002).

In another Caucasian studies, it was interesting to note that the distribution of the *GSTs* genotypes among the US and the German populations were very similar. For instance, the distribution of *GSTM1* null, *GSTT1* null and *GSTP1* (val/val) genotypes among the German were 47.3%, 18.5% and 11.3% respectively (Schneider *et al*., 2004). Meanwhile among the US population, the genotypic distribution was 47.4% (*GSTM1* null), 18.6% (*GSTT1* null) and 13.1% for (*GTSP1* val/val*)* genotypes (Cote *et al*., 2005).

The only study done in Singapore this region that uses some of the subjects from Malaysia, found that the proportions of Chinese, Malays and Indians with the *GSTT1* null genotype were 58%, 38% and 16% respectively. Meanwhile, the frequency of the combined *GSTM1* and *GSTT1* null genotype among Chinese, Malays and Indians were 37%, 22% and 5% respectively (Lee *et al*., 1995).

Among the Chinese in China, the frequency of *GSTM1* null genotype was 54.3% and the frequency of the *GSTP1* ile/ile (wild-type), ile/val and val/val genotype was 60.7%, 35.2% and 4.1%, respectively (Zhong *et al*., 2006).

In Thailand, the distribution of the *GSTM1* null genotype in their population reported to be 30.2% which was similar with the study done in Southern India (Kietthubthew *et al*., 2001). Meanwhile, almost half of the Thai population (47.2%) consists of individuals with *GSTT1* null genotypes.

In the South India population study, 30.4% and 16.8% of the individuals lacked (null) of the *GSTM1* and *GSTT1* gene respectively. Meanwhile, 4.6% of these people lacked (null) of both the *GSTM1* and *GSTT1* (Naveen *et al*., 2004). In another study from the same region South India population by V *et al*., (2006), the *GSTM1* and *GSTT1* null genotype frequencies were found to be 22.4% and 17.6% respectively. The *GSTP1* genotype frequency was 58.4% for ile/ile – wild type, 38.4% for ile/val and 3.1% for val/val (V et al., 2006).

Among Korean males, 53.8% of the individuals had the *GSTM1* null genotype and 54.3% had the *GSTT1* null genotype. The genotypic distribution of *GSTP1* was 68.4% for ile/ile – wild type, 29.1% for ile/val and 2.5% for val/val. Twenty-nine percent had the null genotype for both *GSTM1/GSTT1* genes. As for the combination of *GSTM1/GSTT1/GSTP1*, the wild type frequency was 14.9% (Cho *et al*., 2006).

Table 2.2: Summary of distribution on GSTM1, GSTT1 and GSTP1 genotypes in normal population

Population	Sample size, n	*GSTM1* null (%)	*GSTT1* null (%)	*GSTM1* & *GSTT1* null (%)	*GSTP1*			*GSTM1, GSTT1* & *GSTP1* wild-type (%)	Reference
					ile/ile (wild-type) (%)	ile/val (%)	val/val (%)		
Caucasian									
Brazilian	591	42.1	25.4	9.8	49.7	38.1	12.2	-	Rossini *et al*., (2002)
USA	290	47.4	18.6	-	37.9	49.0	13.1	-	Cote *et al*., (2005)
Germany	622	47.3	18.5	-	47.9	40.8	11.3	-	Schneider *et al*., (2004)
Italy	100	56.0	31.0	-	-	-	-	-	Capoluongo *et al*., (2006)
Netherlands	207	51.7	20.3	11.1	-	-	-	-	Oude Ophuis *et al*., (1997)
France	172	52.3	15.7	-	50.0	37.2	12.8	-	Jourenkova-Mironova *et al*., (1999)
Asian									
Singapore									Lee *et al*., (1995)
Malay	167	-	38.0	22.0	-	-	-	-	
Chinese	187	-	58.0	37.0	-	-	-	-	
Indian	152	-	16.0	5.0	-	-	-	-	
China	196	54.3	-	-	60.7	35.2	4.1	-	Zhong *et al*., (2006)

- Not Reported

Table 2.2: Summary of distribution on *GSTM1, GSTT1* and *GSTP1* genotypes in normal population (continue)

Population	Sample size, n	*GSTM1* null (%)	*GSTT1* null (%)	*GSTM1* & *GSTT1* null (%)	*GSTP1* ile/ile (wild-type) (%)	*GSTP1* ile/val (%)	*GSTP1* val/val (%)	*GSTM1, GSTT1* & *GSTP1* wild-type (%)	Reference
Asian									
Thailand	53	30.2	47.2	-	-	-	-	-	Kietthubthew *et al*., (2001)
South Indian	517	30.4	16.8	4.6	-	-	-	-	Naveen *et al*., (2004)
South Indian	255	22.4	17.6	-	58.4	38.4	3.1	-	V *et al*., (2006)
Korean (male)	1051	53.8	54.3	29.0	68.4	29.1	2.5	14.9	Cho *et al*., (2005)

- Not Reported

2.5 Dietary ITCs, GSTs and cancer prevention

2.5.1 Metabolism of carcinogen

Both genetic and environmental factors are involved in the development of cancer. The environment-gene interaction on carcinogenesis has been well demonstrated by phase I and II enzymes that are involved in the metabolism of carcinogens. These enzymes are often termed xenobiotic metabolizing enzymes (XMEs) and found mainly in the liver and also in the upper aerodigestive tract mucosa. Some of these enzymes are polymorphic in genotypes, with corresponding variation in their activities (Hung *et al*., 1997). Besides, some of these XMEs also strongly influence the individual's biological responses to carcinogens by formation of DNA adducts. Hence, certain XME genotype may increase individual susceptibility to cancer through erroneous carcinogen exposure (Kumar and Zain, 2004).

In our human metabolism pathway, phase I involves the 'activation' of the carcinogen by oxygenation. This is mainly performed through cytochrome P450 enzymes encoded by the CYP gene superfamily. Phase II enzymes is the detoxification step, in which the 'activated' carcinogen is rendered more hydrophilic, thus it is easily excretable (Miller *et al*., 2003). One of the most important detoxification enzyme systems is the glutathione s-transferase (GST) family of enzymes. Human *GSTs* enzymes can be subdivided into five main classes, alpha (α), **m**u (μ), **p**i (π), **t**heta (θ) and **z**eta (ζ) (Cho *et al*., 2006). However, for the purpose of this study, *GSTM1, GSTT1* and *GSTP1* genes would be the main focus in the discussion.

An example of the involvement of phase I and II metabolism pathway are as follows: Metabolism of carcinogens such as benzo(α)pyrene involves a balance of activation steps that produces reactive intermediates and detoxification steps that produce water-soluble, excretable compounds. Activation is often mediated by the cytochrome P450 pathway and can result in the formation products known as DNA adducts. A person who does not have the ability to produce the GSTM1 enzyme potentially accumulates more DNA adducts through their inefficiency at excreting activated carcinogens such as 7,8-diol-9,10-epoxide (Geisler and Olshan, 2001). Thus, this individual may pose to be highly susceptible to cancer risk.

One study by Park *et al*., (1997) showed that the prevalence of the CYP1A1 (ile/val) polymorphism [including both the (ile/val) and (val/val) genotypes] was significantly higher in cases as compared to controls (17.6% versus 7.6%, respectively; crude odds ratio 2.6, 95% CI 1.2 – 5.7). These results suggest that individuals with CYP1A1 exon 7 ile/val polymorphism genotypes are at risk for oral cancer, and that this risk may not be influenced by differences in exposure to tobacco smoke (Park *et al*., 1997).

2.5.2 ITCs and GSTs as preventive agents against cancer

Dietary carcinogens such as PAHs, heterocyclic amines (HAs) and nitrosamines require metabolic activation to cause DNA-damage and cancer. The activation of carcinogens is primarily catalysed by phase I enzymes such as cytochrome P450 (Steinkellner *et al*., 2001). Protection can be accomplished by inhibition of activating enzymes and/or by induction of phase II which leads to detoxification and accelerated excretion of carcinogens.

Induction of phase II detoxification enzyme (GSTM1/GSTT1/GSTP1) is a powerful strategy for achieving protection against carcinogenesis, mutagenesis and other form of toxicity of electrophiles and reactive forms of oxygen. Since consumption of large quantities of fruit and vegetables is associated with a striking reduction in the risk of developing a variety of malignancies, it is of interest that a number of edible plants contain substantial quantities of compounds that regulate human enzymes of xenobiotic metabolism (Fahey *et al*., 1997).

Many ITCs, which are available to human subjects mainly through consumption of cruciferous vegetables, demonstrate strong cancer-prevention activity in animal models (Hecht, 1999). Human studies also show an inverse association between consumption of ITC and risk of cancer in several organs. Zhang and Talalay (1998) in their study found that there is in-vitro and in-vivo evidence that ITCs are potent anticarcinogenic compounds. These anticarcinogenic properties of ITCs have been attributed to their ability to alter detoxification pathways (Zhang and Talalay, 1998; Hecht, 1999), leading to decreased activation of procarcinogens and increased excretion of carcinogens.

It has been well established that ITCs can inhibit cancer development through multiple mechanisms (Zhang and Talalay, 1994; Hecht, 2000; Conaway *et al*., 2002; Zhang *et al*., 2007), including:

(i) blocking DNA damage by both inhibition of carcinogen activation of phase I enzymes (mainly cytochrome P450) and detoxification of reactive carcinogens through induction of phase II enzymes (e.g. GSTs). The *in vitro* and *in vivo* findings with ITCs are supported by human studies; daily intake of 300g

Brussels sprouts or 300g red cabbage induced plasma GST (Steinkellner *et al*., 2001).

(ii) reducing oxidative stress by elevating and maintaining cellular antioxidants

(iii) inhibiting cell proliferation

Other effects, including anti-inflammation, anti-infection, and perhaps induction of differentiation also have been observed with some ITCs and may contribute to the overall cancer-preventive effects of these compounds. The ability of ITCs to simultaneously modulate multiple cellular targets involved in cancer development is of significant importance (Zhang, 2004). These findings shed new light on the mechanism of action of ITC and indicate that ITC may be useful both as cancer-preventive and therapeutic agents.

Specifically, ITCs are tasked to inhibit phase I activating enzymes, and induce phase II detoxification enzymes in various target tissues (Zhang and Talalay, 1994; Fahey *et al*., 1997; Hecht, 1999). ITCs, in particular exert their effects through the latter pathway. Induction of phase II detoxification enzymes reduces exposure of the target tissue to DNA damage, thus exerting a 'blocking effect' on the initiation stage of chemical carcinogenesis (Steinkellner *et al*., 2001; Rouzaud *et al*., 2004). ITCs and other phase II enzyme inducers can also act as 'suppressing agents' during the post-initiation stage of carcinogenesis by promoting apoptosis, and suppressing malignant transformation, possibly through their effect on the cellular glutathione pool (Grubben *et al*., 2001; Kirlin *et al*., 1999; Bonnesen *et al*., 2001). This dual action is thought to reduce the production of electrophilic intermediates with carcinogenic activity and to enhance the detoxification and clearance of carcinogens.

ITCs are not only inducers of phase II enzymes but are also substrates for GSTs, which are phase II enzymes (Zhang *et al*., 1995). The enzyme-catalysed nucleophilic attack of the sulfhydryl group of glutathione on the central carbon of the ITC group results in the formation of glutathione dithiocarbamates that are modified by a sequence of enzymatic reactions, leading ultimately to the formation of N-acetlycysteine dithiocarbamates (also known as mercapturic acid). The glutathione adduct and its four sequential metabolic products are excreted in the urine, but the N-acetylcysteine mercapturic acids predominate (Shapiro *et al*., 1998). On the other hand, the GST enzymes also constitute an important part of cellular defense against reactive carcinogens or oxidants. Their protective functions are achieved by formation of polar (water soluble) conjugates of electrophile carcinogens that are easily excreted in the urine or bile, thus reducing the effective body burden of the carcinogen (Conaway *et al*., 2002; Zhang, 2004).

As an initial reaction, *GSTs* catalyze the rather slow conjugation between GSH with the electrophiles, including genotoxic chemicals and ITCs (Conaway *et al*., 2002). Some ITCs are readily conjugated by human *GSTM1* and *GSTP1* but are slowly conjugated by other forms (Meyer *et al*., 1995; Zhang *et al*., 1995). The rates of catalytic conjugation of ITCs by various forms of *GST* depended very much on the structure of the ITCs. It is conceivable that ITC, as an inducer of *GST*, facilitates its own excretion by increasing its rate of conjugation with GSH (IARC, 2004).

In a previous study by Zhang and Talalay, (1998) they showed that many ITCs rapidly accumulate in cells to very high concentrations (up to millimolar levels), and the accumulations appeared to play a critical role in determining their activites in inducing anticarcinogenic phase II enzymes. Subsequent studies showed that ITCs were principally

accumulated as GSH conjugates in cells and that cellular GSH might be the major driving force for ITC accumulation by undergoing rapid conjugation with the entering ITCs. Elevating cellular GSH levels also resulted in nearly proportional increases in cellular ITC uptake. Interestingly, lipophilicity of ITCs did not seem to influence ITC uptake by cells. Taken together, it is concluded that ITCs are taken up by cells predominantly, if not entirely, through GSH conjugation reactions in cells, and that cellular GST promotes ITC uptake by enhancing the conjugation reaction (Zhang, 2001).

2.6 Dietary ITCs, GSTs polymorphisms and cancer risk

2.6.1 Dietary ITCs and cancer risk

Dietary ITCs may play an important role in the prevention of human cancers (Jiao *et al*., 1998). Epidemiological study provides evidence that the consumption of cruciferous vegetables protects against cancer more effectively than the total intake of fruits and vegetables.

Epidemiologic studies have demonstrated inverse associations between crucifer intake and the incidence of lung, breast, bladder, prostate, stomach and colon cancer (Verhoeven *et al*., 1996). Prospective dietary assessment of 628 men diagnosed with prostate cancer found that increasing crucifer intake from 1 to 3 or more servings per week resulted in a 41% decreased apparent risk (Cohen *et al*., 2000). A 10-year cohort study of 47,909 men reported that increased crucifer intake, but not fruits and other vegetables, was associated with decreased risk for bladder cancer (Michaud *et al*., 1999). Verhoeven and

co-workers also reviewed the results of 7 cohort studies and 87 case-control studies and reported that 67% of the case control studies found inverse associations between total crucifer intake and cancer risk (Verhoeven *et al*., 1996). Inverse associations between cancer risk and intakes of cabbage, broccoli, cauliflower or brussel sprouts were noted in 70%, 56%, 67% and 29% of the control studies, respectively. The cohort studies showed inverse associations between intakes of cabbage, cauliflower or broccoli and risks for lung cancer; between total crucifer intake and risk for stomach cancer; and between broccoli intake and risk for all cancers. Although some reports have attributed to the protective activities of cruciferous vegetables to their glucosinolate content, other phytochemicals and constituents, i.e. carotinoids, vitamins, folic acid, selenium, dietary fiber, coumarins, flavonoids etc. may also contribute.

In another study, human subjects with detectable urinary excretion of total ITCs were found to have much lower incidence of lung cancer (smoking-adjusted relative risk = 0.65) than those with undetectable urinary ITCs (London *et al*., 2000). Despite the relationship between exposure to ITCs and human cancer risk being reported, not many have studied the effect of dietary ITCs on oral cancer. Only one study by Nair and Bartsch (2001) had identified ITC as the possible chemopreventive agents for cancer of the oral cavity.

2.6.2 GSTs polymorphisms and cancer risk

The impact of genetic polymorphisms in *GSTM1*, *GSTT1* and *GSTP1* on the susceptibility to cancer has received particular interest since these enzymes play a central role in detoxification of major classes of tobacco carcinogens. There were many studies

done on the *GSTs* polymorphisms and the risk of cancers such as lung, esophageal, cervical, breast and bladder cancer. While some of these studies showed that *GSTs* polymorphisms could be the genetic determinant in the development of cancers, others find it otherwise. Table 2.3 summarizes the *GSTs* polymorphisms and cancer risks.

Among the Caucasians, some studies have shown that there was no association between *GSTM1, GSTT1* and/or *GSTP1* polymorphism and risk of lung cancer (Nazar-Stewart *et al*., 2003; Wang *et al*., 2003; Schneider *et al*., 2004; Cote *et al*., 2005; Wenzlaff *et al*., 2005). However, Miller *et al*., (2003) in his study found that *GSTP1* polymorphism increased the lung cancer risk associated with pack-years of smoking. Meanwhile, Sweeney *et al*., (2003) also discovered that *GSTM1* null genotype confers susceptibility to lung cancer, although there was no similar association found between *GSTT1* and *GSTP1* polymorphism.

In the Asian region, a study among the non-smoking Chinese in Hong Kong showed that *GSTT1* null genotype was associated with an increased risk for lung cancer (OR 1.69, 95% CI 1.12-2.56) (Chan-Yeung *et al*., 2004). On the other hand, the population in the rural Thailand reported by Pisani *et al*., (2006) showed that *GSTM1* null had no effect on the risk of lung cancer.

A study done in China by Tan *et al*., (2000) demonstrated that *GSTM1* null genotype could be a possible genetic determinant in the development of squamous cell carcinoma of the esophagus. On the contrary, Jain *et al*., (2006) reported that genetic polymorphisms of *GSTM1, GSTT1* and *GSTP1* were not associated with higher risk of esophageal cancer among the North Indian population.

There was no overall association of the *GSTM1* and *GSTT1* null genotype with breast cancer risk, but the *GSTP1* polymorphism (val/val) may be significant to breast cancer risk in the Asian populations (Egan *et al*., 2004). In another study done by Sobti *et al*., (2006) found that *GSTM1*, *GSTT1* and *GSTP1* was associated with an increase risk of developing cervix cancer among the passive smokers. Meanwhile, Srivastava *et al*., (2005) demonstrated that only *GSTP1* (val/val) polymorphism was a strong predisposition risk factor for bladder cancer (OR 7.12, 95% CI 3.14-16.16).

Table 2.3: Summary of GSTM1, GSTT1 and GSTP1 polymorphisms and cancers risk

Population	Sample size, n		Site	*GSTM1* null		*GSTT1* null		*GSTP1* polymorphism (ile/val and/or val/val)		Reference
	Case	Control		↑ risk	NA	↑ risk	NA	↑ risk	NA	
Caucasian										
[b]USA	274	501	Lung	-	X	-	X	-	X	Nazar-Stewart *et al*., (2003)
[b]USA	582	600	Lung	-	X	-	X	-	X	Wang *et al*., (2003)
[b]Germany	446	662	Lung	-	X	-	X	-	X	Schneider *et al*., (2004)
[b]USA	350	410	Lung	-	X	-	X	-	X	Cote *et al*., (2005)
[a]USA	166	181	Lung	-	X	-	X	-	X	Wenzlaff *et al*., (2005)
[b]USA	1042	1161	Lung	-	-	-	-	X	-	Miller *et al*., (2003)
[f]USA	253	-	Lung	X	-	-	X	-	X	Sweeney *et al*., (2003)
Asian										
[a]Hong Kong	229	197	Lung	-	X	X	-	-	X	Chan-Yeung *et al*., (2004)
[e]Thailand	211	211	Lung	-	X	-	-	-	-	Pisani *et al*., (2006)
[b]China	150	150	Esophagus	X	-	-	X	-	X	Tan *et al*., (2000)
[c]North India	100	137	Esophagus	-	X	-	X	-	X	Jain *et al*., (2006)
[d]India	103	103	Cervix	X	-	X	-	X	-	Sobti *et al*., (2006)
[e]China	1144	1221	Breast	-	X	-	X	X	-	Egan *et al*., (2004)
[e]North India	106	370	Bladder	-	X	-	X	X	-	Srivastava *et al*., (2005)

NA: no association; [a]never-smoker; [b]smoker; [c]mixed habits – smoker & drinker; [d]passive smoker, [e]unavailable
- Not Reported

2.6.3 GSTs polymorphisms and oral cancer risk

Polymorphisms in the gene encoding the *GSTs* metabolizing enzyme have previously been associated with susceptibility to various cancers. Many studies have looked into the roles of *GSTs* polymorphisms with the oral cancer susceptibility, but the findings have been inconsistent. The following table 2.4 gives an overview of the *GST* polymorphisms and the risk of oral cancer.

Some studies have shown that among the Caucasians, there was no association between *GSTM1, GSTT1* and/or *GSTP1* polymorphism and susceptibility to oral cancer risk (Park *et al*., 1997; Oude-Ophuis *et al*., 1998; Olshan *et al*., 2000; Hahn *et al*., 2002; Gronau *et al*., 2003). Meanwhile, no association was also observed between the *GSTM1* and *GSTT1* null genotypes and oral cancer risk in Asian namely Taiwan, India and Japan (Hung *et al*., 1997; Sreelekha *et al*., 2001; Sugimura *et al*., 2006).

However, in a study from Italian Lazio region, Capoluongo *et al*., (2006) found that genetic alteration of *GSTM1* detoxifying enzyme as a risk factor for the development of head and neck cancer (HNSCC). A meta-analysis done by Ye *et al*., (2004) also supported the hypothesis that *GSTM1* (OR 1.27, 95% CI 1.13-1.42) and *GSTT1* (OR 1.14, 95% CI 1.00-1.31) were important risk factors for HNSCC and suggested that *GSTM1* and *GSTT1* deficiency may have an effect on the risk of developing HNSCC. On another note, Morita *et al*., (1999) and Cho *et al*., (2006) reported that polymorphism (val/val) of *GSTP1* were also associated with increased risk of HNSCC.

Several studies especially from the Asian populations strongly suggest that *GSTM1* null genotype as a risk factor for the development of oral cancer. A study done by Buch *et al*., (2002) among the Indians found that individuals with *GSTM1* null genotype may have 3 times the risk of developing oral cancer (OR 3.2, 95% CI 2.4-4.3). Similar risk was also observed in both Japanese (OR 2.2, 95% CI 1.4-3.6) and the Thailand population (OR 2.6, 95% CI 1.04-6.50) (Sato *et al*., 1999; Kietthubthew *et al*., 2001). Another study by Cha *et al*., (2007) also supported that *GSTM1* null genotype was highly susceptible and closely associated with increased risk of oral cancer in Koreans. *GSTM1* null polymorphism has also been linked with an increased risk of oral cancer among Japanese and African-American smokers (Trizna *et al*., 1995; Kihara *et al*., 1997). These studies are consistent with the previously observed metabolic activity of *GSTM1* towards PAHs since these carcinogens are abundant in tobacco smoke (Ketterer *et al*., 1998).

Previous study by Jourenkova-Mironova *et al*., (1999) and Sharma *et al*., (2006) among the Caucasian smokers suggested that *GSTP1* and *GSTT1* gene polymorphisms modulate susceptibility to smoking-related cancers of the oral cavity. Sikdar *et al*., (2004) and Park *et al*., (1999) found that polymorphism (val/val) of *GSTP1* was associated with oral cancer risk among Indian tobacco smokers and Caucasians light smokers respectively. On another note, Hashibe *et al*., (2003) found that the combination of *GSTM1* null, *GSTT1* null and *GSTP1* polymorphism genotypes conferring an OR of 2.06 (95% CI 1.11-3.81) were associated with higher risk of oral cancer.

Because these carcinogen-metabolizing enzymes may be among numerous genes involved in the multistage pathway of cancer, they are expected to be modest to moderate risk factors that may be difficult to detect. However, even modest single gene effects on

cancer risk are of biological and medical importance because of the possibility of identifying, under multigenic models, high-risk individuals for target prevention activities (Hashibe *et al*., 2003).

Table 2.4: Summary of GSTM1, GSTT1 and GSTP1 polymorphism and oral cancer risk

Country	Sample size, n		Site	Population	*GSTM1* null		*GSTT1* null		*GSTP1* polymorphism (ile/val and/or val/val)		Reference
	Case	Control			↑ risk	NA	↑ risk	NA	↑ risk	NA	
Caucasian											
[b]USA	186	42	Oral	White	X	-	-	X	-	-	Trizna *et al*., (1995)
[b]USA	135	135	Oral	White	-	X	-	-	-	-	Park *et al*., (1997)
[a]USA	104	175	Oral	White	-	-	-	-	X	-	Park *et al*., (1999)
	53	85		Black	-	-	-	-	-	-	
[a]USA	112	174	Oral	White	-	X	-	X	-	X	Olshan *et al*., (2000)
	70	28		Black	-	-	-	-	-	-	
[b]Netherlands	185	207	Oral	Caucasian	-	X	-	X	-	-	Oude-Ophuis *et al*., (1998)
[b]Germany	94	92	Oral	Caucasian	-	X	-	-	-	-	Hahn *et al*., (2002)
[b]Germany	73	136	Oral	Caucasian	-	X	-	X	-	-	Gronau *et al*., (2003)
[a]France	121	172	Oral	Caucasian	-	X	X	-	X	-	Jourenkova-Mironova *et al*., (1999)
[b]Italy	80	80	HNSCC	Caucasian	X	-	-	X	-	-	Capoluongo *et al*., (2006)

NA: no association; [a]smoker; [b]unavailable
- Not Reported

Table 2.4: Summary of GSTM1, GSTT1 and GSTP1 polymorphism and oral cancer risk (continue)

Country	Sample size, n		Site	Population	*GSTM1* null		*GSTT1* null		*GSTP1* polymorphism (ile/val and/or val/val)		Reference
	Case	Control			↑ risk	NA	↑ risk	NA	↑ risk	NA	
Asian											
[d]Taiwan	41	123	Oral	Chinese	-	X	-	X	-	-	Hung *et al*., (1997)
[e]India	98	60	Oral	Indian	-	X	-	X	-	-	Sreelekha *et al*., (2001)
[c]Japan	122	241	Oral	Asian	-	X	-	X	-	-	Sugimura *et al*., (2006)
[a]India	40	87	Oral	Indian	-	X	X	-	-	-	Sharma *et al*., (2006)
[a]India	285	426	Oral	Indian	X	-	-	X	-	-	Buch *et al*., (2002)
[a]Japan	142	142	Oral	Asian	X	-	-	-	-	-	Sato *et al*., (1999)
[e]Thailand	50	53	Oral	Buddhist	X	-	-	X	-	-	Kiettubthew *et al*., (2001)
	3	0		Muslim							
[a]Korea	72	221	Oral	Asian	X	-	-	-	-	-	Cha *et al*., (2007)
[a]Japan	158	474	Oral	Asian	X	-	-	-	-	-	Kihara *et al*., (1997)
[a]India	256	259	Oral	Indian	-	X	-	X	X	-	Sikdar *et al*., (2004)
[b]Japan	145	164	HNSCC	Asian	-	X	-	-	X	-	Morita *et al*., (1999)
[a]Korea	294	333	HNSCC	Asian	-	-	-	-	X	-	Cho *et al*., (2006)

NA: no association; [a]smoker; [b]drinker; [c]mixed habits – smoker & drinker; [d]mixed habits – smoker, drinker and chewer; [e]unavailable
- Not Reported

2.6.4 Dietary ITC intake and the association with GSTs polymorphisms and cancer risk

The relationship between intake of ITCs and cancer appears to be complex, and there are individual differences in response to ITCs, which depend on variations in biotransformation enzymes (Ketterer, 1998). This gene-environment interaction has been best studied in relation to the genetic polymorphisms that affect GST expression (Hayes and Strange, 2000). GST catalyses the conjugation of glutathione to ITCs, and ITCs are among the substrates most rapidly conjugated by GST (Kolm *et al*., 1995). Because GSTs are also known to metabolize ITCs, this pathway results in the formation of N-acetylcysteine conjugates, which are excreted in the urine (Brusewitz, 1977 and Jiao, 1994). Based on the biological interaction between GST and ITCs, the beneficial effect of ITC is therefore dependent in part on the presence or absence of GST activity. Individuals with low activity or null for GST will metabolize these compounds at a slower rate and therefore less readily conjugate and excrete ITC. Thus, this would be expected to have greater amounts of ITC at the tissue level, and hence would experience a greater protective effect (Seow *et al*., 2005).

Epidemiological studies have been designed to specifically evaluate dietary ITC consumption as a protective factor in human cancer. Table 2.6 summarizes dietary ITC intake and the association with *GSTs* polymorphisms and cancer risk. In one study, cruciferous vegetable consumption of 246 Singapore Chinese, 111 men and 135 women ages 45-74, was evaluated using cyclocondensation assay for daily total urinary ITC excretion (Jiao *et al*., 1998). It was proposed that *GSTM1* null individuals would excrete ITCs more slowly because of insufficient activities of *GSTs*, but in this study there was no

significant difference ($p = 0.61$) in urinary excretion of ITCs between *GSTM1* null and *GSTM1* non-null subjects. Urinary excretion of ITCs was, however, significantly higher among *GSTT1* non-null subjects relative to *GSTT1* null individuals ($p = 0.006$). The strength of the association was highly dependent on the level of cruciferous vegetable consumption by study subjects.

Another study evaluated the direct relationship between total ITCs in urine, *GST* genotype, and subsequent risk for lung cancer among 232 incident cases of lung cancer and 710 matched controls from a cohort of 18,244 men in Shanghai, China (London *et al*., 2000). Individuals with ITCs in the urine were at a decreased risk for lung cancer. Interestingly, the protective effect of dietary ITCs was more pronounced in persons with the homozygous *GSTM1* null genotype (relative risk 0.36, 95% CI 0.20-0.63) and was particularly strong in subjects with deletion of both *GSTM1* and *GSTT1* (relative risk 0.28, 95% CI 0.13-0.57). It was hypothesized that the reduced rate of excretion of ITCs in persons lacking the specific genotype(s) for *GSTs* that conjugate ITCs may result in higher levels of ITCs in the body, thus, enhanced the chemopreventive effects of dietary ITCs. The results provide the first direct evidence that links dietary ITCs to reduced incidence of lung cancer in humans.

A subsequent case-control study involving 503 cases of lung cancer and 465 controls of American Whites was conducted by Spitz and colleagues (2000). Dietary ITCs were estimated on the basis of a questionnaire. The mean total ITC intakes were estimated to be 0.47 ± 0.51mg/1000kcal for the cases and 0.58 ± 0.84mg/1000kcal for the control. Cases of lung cancer reported a significantly lower ITC intake per day compared with controls ($p = 0.009$). Although there was no discernable effect on lung cancer incidence

associated with the *GSTM1* null genotype, there was a statistically significant OR of 1.41 associated with the *GSTT1* null genotype. For current smokers with the *GSTT1* null genotype, the OR with low ITC intake was 3.19 (95% CI 1.54-6.62); the comparable OR with low ITC intake for both *GSTM1* null and *GSTT1* null genotypes was 5.45 (95% 1.72-17.22). However, these effects were not demonstrated for *GSTM1* null, *GSTT1* null and combined *GSTM1* and *GSTT1* null genotypes with the high ITC intake. The OR for *GSTM1* null, *GSTT1* null and combined *GSTM1* and *GSTT1* null genotypes with the high ITC intake were 1.55 (95% CI 0.79 – 3.04), 1.31 (95% CI 0.60 – 2.85) and 1.09 (0.38 – 3.14), respectively and found to be not statistically significant.

Recently, another hospital-based case-control study evaluated the association between dietary ITC intake, *GSTM1* and *GSTT1* polymorphisms, lung cancer risk in 420 Singapore Chinese women (Zhao *et al*., 2001). Dietary ITC intakes were estimated from a food frequency questionnaire and were relatively more objective because samples of the consumed vegetables were quantified for total ITC contents. Higher weekly intake of ITCs (above the control median value of 53.0μmol) reduced the risk of lung cancer to a greater extent in smokers (OR 0.31, 95% CI 0.10-0.98) than in non-smokers (OR 0.70, 95% CI 0.45-1.11). Among non-smokers with *GSTM1* null genotype, higher intake of ITCs significantly reduced the risk of lung cancer (OR 0.54, 95% CI 0.30-0.95); the effect was not observed in subjects with detectable *GSTM1* (OR 1.07, 95% CI 0.50-2.29). The results were consistent with the hypothesis that ITC intake is inversely related to risk of lung cancer, but that among non-smokers, the effect is primarily confined to *GSTM1* null individuals. Thus, three well-performed studies indicate that lung cancer risk is reduced by dietary sources of ITCs, and that person with the *GSTM1* null and *GSTT1* null genotypes clearly benefit more extensively from diets rich in ITCs.

The protective effects of dietary ITCs against colon cancer have also been reported. Seow *et al*., (2002) compared 213 incident cases of colorectal with 1194 controls. The cases were identified through the population-based Singapore Cancer Registry involving 63,257 men and women, who were enrolled from 1993 to 1998. Information on dietary ITC intake was collected at the recruitment via a semi-quantitative food frequency questionnaire. Dietary ITC intake was slightly lower in the cases (mean 5.4µmol/1000kcal) than the controls (6.0µmol/1000kcal), but the difference was not significant. Although there were no overall associations between *GSTM1, GSTT1* or *GSTP1* genotypes and colorectal cancer risk, there was a 57% reduction in risk among high (greater than median dietary ITC intake of 5.2µmol/1000kcal) versus low (less than/equal to median) consumers of ITC in individuals with both *GSTM1* and *GSTT1* null genotypes (OR 0.43), in particular for colon cancer (OR 0.31).

Another study by Wang *et al*., (2004) on the dietary intake of cruciferous vegetables, *GSTs* polymorphisms and lung cancer risk in a Caucasian population found that higher intakes of cruciferous vegetables reduced lung cancer risk among *GSTM1* present individuals (OR 0.61, 95% CI 0.39-0.95) but not among *GSTM1* null individuals (OR 1.15, 95% CI 0.78-1.68). No significant interactions were observed for *GSTT1* or the combined *GSTM1/T1* genotype.

Table 2.5: Summary of dietary ITC intake and the association with GSTs polymorphisms and cancer risk

Population	Site	High dietary ITC intake								Reference
		GSTM1 null		*GSTT1* null		*GSTM1 & GSTT1* null		*GSTP1* polymorphism		
		↓ risk	NA	↓ risk	NA	↓ risk	NA	↓ risk	NA	
Caucasian										
USA	Lung		X	X						Spitz *et al*., (2000)
USA	Lung		X		X		X			Wang *et al*., (2004)
Asian										
China	Lung	X				X				London *et al*., (2000)
Singapore	Lung	X								Zhao *et al*., (2001)
Singapore	Colorectal		X		X	X			X	Seow *et al*., (2002)

NA: no association