

No germline mutations in *CDKN2A* (*p16*) in patients with squamous cell cancer of the head and neck and second primary tumours

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Summary There is increasing evidence that predisposition to some cancers has a genetic component. There is a high incidence of loss of heterozygosity on chromosome 9, in the region of tumour suppressor gene, *CDKN2A* (also known as *p16*), in sporadic squamous cell cancer of the head and neck (SCCHN). To investigate the possibility that *CDKN2A* may be involved in the inherited susceptibility to SCCHN, the 3 coding exons of *CDKN2A* were sequenced in 40 patients who had developed a second primary cancer after an index squamous cell cancer of the head and neck. No mutations were found and we conclude that *CDKN2A* mutations do not play a major role in cancer susceptibility in this group. © 2001 Cancer Research Campaign

Keywords: squamous cell cancer of the head and neck; *CDKN2A* (*p16*) gene; mutation

Squamous cell cancer of the head and neck (SCCHN) is a disease associated with major morbidity and mortality. It is the fifth most common cancer worldwide and the incidence of SCCHN is increasing in developing countries (Macfarlane et al, 1994; Parkin et al, 1985). The epidemiology of SCCHN is complex due to the multigenic nature of the disease and the number of potential environmental agents to which individuals may have been exposed. It is clear that the major aetiological agents are tobacco and alcohol exposure (Maier et al, 1992; Vokes et al, 1993). Alcohol and tobacco exposure increase the risk of SCCHN in a dose-dependent and independent manner. Other risk factors include nutrition, occupation, viral infection and poor dentition (Maier et al, 1991; Muscat and Wynder, 1992). These risk factors do not, however, adequately explain all cancer cases. Until recently, little attention has been paid to possible hereditary factors in SCCHN. However, 3 recent case-control studies have shown a relative risk (RR) for SCCHN of 3.5–3.8 in association with a family history of cancer of the upper aerodigestive tract (UADT) and/or lung cancer (Copper et al, 1995; Foulkes et al, 1995, 1996).

Despite therapeutic advances in the treatment of SCCHN, the overall survival from the disease has remained unchanged over the last 20 years. A contributory factor to this is that patients with successfully treated early-stage SCCHN have a high incidence of second primary cancers of SCCHN or UADT (Day and Blot, 1992). Generally, the presence of multiple primary cancers in an individual is a sign that there may be an increased hereditary predisposition to cancer in that person. Inter-individual differences in susceptibility to second tumours could be accounted for by

genetic factors (Trizna and Schantz, 1992; Bongers et al, 1996). Individuals with a second primary SCCHN are more likely to have a family history of SCCHN than those with only single primaries (Bongers et al, 1996; Foulkes et al, 1996; Morita et al, 1998).

The tumour suppressor gene *CDKN2A* (also known as *p16*) is localized on chromosome 9p21 and is thought to play an important role in the development of some cancers. The gene consists of 3 coding exons, exon 1 (125 bp), exon 2 (307 bp) and exon 3 (12 bp) (Kamb et al, 1994a). It encodes a 16 kDa protein that binds to cyclin-dependent kinase (cdk) 4 and 6 inhibiting their association with cyclin D1 (Serrano et al, 1993). The inhibition of the cyclin D1-cdk4/6 complex activity prevents retinoblastoma protein phosphorylation, which normally leads to the arrest of the cell cycle in the G1/S transition (Serrano et al, 1993). Somatic *CDKN2A* deletions have been documented in melanoma, oesophageal, lung and bladder cancer (Kamb et al, 1994b). Germline *CDKN2A* mutations have been shown to predispose to familial melanoma (Hussussian et al, 1994; Kamb et al, 1994b). *CDKN2A* is thought to be an important tumour suppressor gene in the aetiology of SCCHN. Somatic mutations occur in 10% of SCCHN and homozygous deletions occur in approximately 50% (Reed et al, 1996). Inactivation of *CDKN2A* is thought to be an early event in the development of SCCHN. In families with familial melanoma, an association has been reported with SCCHN and pancreatic carcinoma (Whelan et al, 1995). In one study of 67 cancer prone families, a germ-line mutation in *CDKN2A* (M531) was identified in a woman with multiple primary tumours, including SCCHN. Her grandmother was reported to have an oral cancer (Sun et al, 1997).

The aim of this study was to investigate whether germline *CDKN2A* mutations contributed to the postulated increased

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susceptibility of tumour development amongst patients who had an index SCCHN followed by a second cancer.

MATERIALS AND METHODS

Patient selection

Individuals with index SCCHN who had developed a second primary cancer in accordance with the criteria defined by Hong et al (1990) were identified for the study. An SPT is defined as occurring more than 3 years from the original diagnosis or is separated by greater than 2 cm of entirely normal epithelium. If the SPT occurs in the lung it must be solitary and occur greater than 3 years from the original diagnosis. If an SPT is of different histology these criteria do not apply. An SPT can occur anywhere and is not just limited to the UADT. Venous blood samples in EDTA were obtained from 40 patients with histologically proven SCCHN and an SPT. The patients were participants in a multi-centre molecular epidemiology study assessing the possible underlying genetic mechanisms in head and neck cancer. Clinical and epidemiological characteristics were recorded. A detailed first-degree family history pedigree was obtained from each participant. All blood samples were obtained with written informed consent and ethical approval from participating centres.

Patient characteristics

24 of the patients were male and 16 female. The mean age at initial presentation was 61.3 years (+/-9.8). All had histologically proven SCCHN as an index tumour. The predominant primary site was oral cavity (22 patients) followed by larynx (12), hypopharynx (4) and oropharynx (2). Of the second cancers, 24 were SCCHN, 7 prostate adenocarcinoma, 3 lung SCC, 2 oesophageal SCC, 2 melanoma, 1 colon adenocarcinoma and 1 chronic granulocytic leukaemia. 3 patients had 3 primaries in total; 2 had further SCCHN and 1 developed adenocarcinoma of the lung.

DNA extraction

Peripheral blood samples (20 ml) were collected from 40 patients with SPT and stored in EDTA at -70°C. DNA extraction was undertaken by the method of Kunkel et al (1977) with some modifications. 4 volumes of water were initially added to whole blood. The phenol-chloroform extraction step was not included and the salting out procedure of Miller et al (1988) was used to clean and retrieve the DNA. DNA was washed in 70% alcohol and dried before dissolving in 0.3 ml of water.

Polymerase chain reaction (PCR)

Sample DNA was added to a reaction mix containing 10X buffer (Perkin Elmer), 1.5 mmol MgCl₂ for exon 1 and 2, 1.25 mmol for exon 3, 0.8 mM dNTPs, 1.0 µM primer and 0.4 µl of AmpliTaq Gold (Perkin Elmer), and 5% DMSO. The primer sequences used were as described by Kamb et al (1994b) and Hussussian et al (1994). The total reaction was made up to 50 µl with water. The tubes were topped with mineral oil (Sigma) and cycled in a Hybaid thermocycler. Thermocycling for exon 1 and 2 was 95°C for 9 min; 94°C for 1 min, 60–55°C for 1 min touchdown, 72°C for 1 min – 11 cycles; 94°C for 1 min, 55°C for 1 min, 72°C for 1 min – 25 cycles; 72°C for 10 min. Thermocycling for exon 3 was 95°C for 5

min; 95°C for 1 min, 55–50°C for 1 min touchdown, 72°C for 1 min – 11 cycles; 95°C for 1 min, 50°C for 1 min, 72°C for 1 min – 34 cycles; 72°C for 10 min (adapted from Harland et al 1997). A 5 µl aliquot of PCR reaction mix was run on 2% agarose gel to check for presence of the required product. PCR products were purified before dye terminator sequencing according to Hamoudi et al 1996. The DNA was dissolved in 15 µl of water and an aliquot was run on 2% agarose gel to check presence and purity of the product. A positive control was included in the sample set, a known mutation in exon 3 (supplied by Harland et al, 1997).

Cycle sequencing

PCR products were sequenced directly using an ABI prism dye terminator cycle sequencing reaction kit. Thermocycling was 96°C for 1 min; 96°C for 30 s, 50°C for 15 s, 60°C for 60 s for 25 cycles. The extension products were added to 3 M sodium acetate, precipitated with absolute alcohol and centrifuged. The pellet was washed with 70% alcohol, dried and then stored at -20°C.

Automated sequencing

Exons 1, 2 and 3 of CDKN2A were sequenced in both the forward and reverse direction. Sequencing was conducted on a 6% polyacrylamide denaturing gel in a 1X TBE (Tris borate buffer) using an ABI 377 automated fluorescent DNA sequencer. The DNA pellet was dissolved in 4 µl of formamide and loading dye. The data were stored using DNA Sequencing Analysis Software. On completion of the run the data were analysed using Sequence Navigator software (ABI).

RESULTS AND DISCUSSION

Evidence is increasing that inter-individual differences in cancer susceptibility may be genetically determined. This genetic component in diseases such as SCCHN is likely to be multifactorial and will include alterations in germline tumour suppressor genes, metabolic enzyme function and DNA repair mechanisms. Second primary tumours occur in 10–30% of patients with SCCHN (Franco et al, 1991). As second or multiple primary tumours are associated with several of the hereditary cancer syndromes, Li-Fraumeni syndrome, Bloom syndrome and Fanconi anaemia, (Trizna and Schantz, 1992) and family studies have demonstrated an increased risk of SCCHN amongst relatives of patients with SPT (Bongers et al, 1996; Foulkes et al, 1996; Morita et al, 1998) we assessed the possible role of germline *CDKN2A* mutations in the development of SPT.

Further support for this hypothesis is that *CDKN2A* alterations are common in human cancer and high frequencies of somatic homozygous deletions and mutations are seen in SCCHN (Reed et al, 1996; Miracca et al, 1999). Melanoma kindreds with *CDKN2A* mutations have also been described with cases of SCCHN (Lynch et al, 1981; Whelan et al, 1995; Yarbrough et al, 1996). We screened the full coding sequence of *CDKN2A* for germline mutations in 40 patients with index SCCHN and an SPT. No mutations were identified within the coding region of the gene (incidence 0%: 95% CI 0–8%). A control mutation in exon 3 was detected. One patient was found to have a c→g transition (+32), not previously described, in exon 3. These results suggest that germline mutations in *CDKN2A* do not contribute to the increased cancer susceptibility in this group.

There are a number of issues to consider from the results of this study. In the methodology used, we cannot exclude the possibility that we may have missed a small number of mutations within the *CDKN2A* gene. We would also not have detected changes in the promoter region or introns of *CDKN2A*, which in some way might abrogate the function of the gene. Germline mutations in *CDKN2A* are responsible for the predisposition to melanoma in some families, but not all families that demonstrate 9p21 linkage are found to have mutations. 2 studies of mutation-negative families have demonstrated a 5' UTR variant (G-34T) which creates an aberrant initiation codon (Liu et al, 1999; Harland et al, 2000). This alteration and other non-coding mutations may be important in a small percentage of families.

The selection of patients may have led to a number of factors that could have obscured an underlying genetic effect. Patients were identified for this study who had developed index SCCHN and then an SPT. This resulted in a heterogeneous population in terms of the primary site within the head and neck and subsequent site of the second primary cancer. It is known that younger age of onset of cancer may be associated with an underlying genetic predisposition. However, in this study the average age of development of SCCHN was 61.3 years (+/-9.8 years), which was not lower than observed in a series of single incident cases of SCCHN (62.2 years: SD +/- 13) (data not shown). A family history of cancer is also a marker for possible genetic predisposition. 27 patients had a family history of cancer of which 4 had a relative with SCCHN (Table 1). The male-female ratio was 3:2, which is a higher proportion of females than would be expected in incident single SCCHN, and may reflect gender-based differences in predisposition to second cancers.

The definition of second primary tumours in this study was based on the clinical definition defined by Hong et al (1990). It is likely that some of the patients included in this study had in fact developed a metastasis from their index tumour, and are not genetically predisposed to SPT. Molecular studies have demonstrated solitary lung tumours to be metastases rather than new primaries (Leong et al, 1998) and similarly synchronous and metachronous lesions have been shown to be genetically identical (Worsham et al, 1995; Bedi et al, 1996; Scholes et al, 1998). Results from molecular studies may be incorporated in the definition of SPT in the future.

Other studies assessing patients with multiple primary tumours have been successful in demonstrating germline mutations of tumour suppressor genes. Germline *p53* has been identified in patients with multiple primary tumours and members of cancer-prone families (Wang et al, 1996). Gallo et al (1999) screened 24 consecutive patients with index SCCHN and SPT for *p53* mutations. *p53* germline mutations were identified in the peripheral blood and cancers of 3 patients, 1 was a missense mutation in exon 6 and the other 2 were same-sense polymorphisms with no change

in the amino acid sequence of the p53 protein. Abnormal expression of wild-type p53 protein was seen in normal and pathological tissue from the 2 patients with the p53 polymorphisms.

Another tumour suppressor gene that has been assessed in SCCHN is the *BRCA2* gene. Easton et al (1997) reported an excess of laryngeal cancer (relative risk = 7.67) in a large hereditary breast cancer family. A significant number of cancers of the buccal cavity and pharynx were observed in a study examining a large series of *BRCA2* families from North America and Europe (RR = 2.26; *P* = 0.03) (Breast Cancer Linkage Consortium, 1999). Cancers of the throat and oral cavity were also observed in *BRCA2* carriers in 3 out of 17 French Canadian breast cancer families (Tonin et al, 1998). Hamel et al (1999) assessed 78 cases of SCCHN for founder mutations in *BRCA2* (53 were French Canadian; 25 Ashkenazi Jewish cases). No founder *BRCA2* mutations were observed and therefore the study concluded that screening for *BRCA2*-inherited mutations, as a risk factor for SCCHN, is not warranted.

Although mutations in the *CDKN2A* gene have been reported in kindreds with oral cancer and familial melanoma, we have failed to demonstrate any coding germline *CDKN2A* mutations in 40 patients presenting with index SCCHN and subsequent second primary tumours. Other mechanisms that may increase cancer susceptibility in this group, such as metabolic enzyme polymorphisms and DNA repair enzyme polymorphisms and their relationship with smoking and alcohol exposure are currently being explored.

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Table 1 Age at diagnosis and family history (FH) of SPT patients

Sex	Male (n = 24)	Female (n = 16)
Mean age	years (+/-SD)	
All (n = 40)	61.3 (9.8)	
FH SCCHN (n = 4)	68.5	
FH CA (n = 23)	59.7	
No FH (n = 3)	61.9	

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