Abstract. Nasopharyngeal carcinoma (NPC) is a characteristic tumor displaying epidemiological, genetic and regional distribution properties and is unique by its natural behavior and therapy. Investigation of the molecular and biological changes, gene amplifications and activations that occur during carcinogenesis and progression can provide new insight into the pathology of the disease and may add biological factors that can be used as new prognostic markers. The p53 tumor suppressor gene is the most frequently mutated gene in human cancer. Although point mutations in the p53 gene are observed in nasopharyngeal cancer, the mutation rate is lower than in other tumors. Immunohistochemical studies have shown significant p53 overexpression in NPC material. In this study, p53 protein immunoreactivity was investigated in paraffin sections of primary nasopharyngeal tumors and metastatic cervical lymph nodes and association with clinical and histopathological characteristics was evaluated. Ninety-seven paraffin sections from 81 patients with NPC treated from 1990 to 1996 were examined by immunohistochemistry and were correlated with clinical features and treatment outcome. Among a total of 97 samples, positive staining for p53 protein was observed in 83 (85.5%) samples while no staining was found in 14 (14.5%) cases. Immunoreactivity was observed in 62 (81.5%) of the primary nasopharyngeal biopsy specimens. The correlation between p53 expression and histological type, stage, age and sex distributions was tested. After statistical analysis according to Chi-square test and Yates’ correction, no significant difference was demonstrated (p>0.05). There was no statistically significant correlation with p53 immunoreactivity and overall and disease-free survival. Although the association between NPC and p53 is not clear, our study confirms that p53 overexpression is present in a considerable subset of patients with NPC.

Nasopharyngeal carcinoma (NPC) is a characteristic tumor displaying epidemiological, genetic and regional distribution properties and is unique by its natural behavior, therapy and association with Epstein Barr Virus (EBV). It is one of the most frequently observed malignancies in Southern China, Southeast Asia and Alaska and the Arctic region. The disease is also frequent in the Mediterranean and North Africa (1).

EBV is observed in all malignant non-keratinizing/undifferentiated NPC (UNPC) cells. Although it is well known that EBV acts in multistage carcinogenesis of UNPC, its exact role is not clear (1-5).

Head and neck cancers associated with tobacco and alcohol usually display squamous cell characteristics. On the other hand, NPC is frequently associated with an undifferentiated morphology and is rich in infiltrative, nonmalignant lymphocytes. Moreover, in contrast to squamous cell head and neck cancer, many NPC cases are observed in late adolescence and middle age (1). Differentiated, keratinized NPC, mostly observed in non-endemic regions, is different from undifferentiated NPC in terms of its epidemiological and genetic characteristics and association with EBV (1,2). Keratinized NPC has similar histological and clinical properties as classical squamous cell head and neck cancer.

Although local control can be achieved due to the radiosensitivity of the tumor, the 5-year survival rates are between 30-65% (1). Investigation of the molecular and biological changes, gene amplifications and activations that occur during carcinogenesis and progression can provide new insights into the pathology of the disease and may add new biological markers that can be used as prognostic factors.

The p53 tumor suppressor gene is the most frequently mutated gene in human cancer (6-8). It is located on the
short arm of chromosome 17 and codes a transcription factor which controls the cell cycle checkpoint. The p53 protein arrests the cell at the G1 checkpoint when genomic damage is present and keeps the cell at the G1-phase of the cell cycle either transiently or permanently until the damage is repaired or causes it to undergo apoptosis (6-10).

The p53 gene may be mutated or absent in tumor cells or may be inactivated by interaction with viral proteins. p53 mutations have been associated with aggressive tumors and adverse prognosis (8,11). It has been suggested that the sensitivity of lymphoma, leukemia or seminoma cells to radiation or chemotherapy is due to the lower frequency of p53 mutations in these tumors. In contrast, a high frequency of mutations in the p53 gene may render carcinoma and sarcoma cells more resistant to radiation and anticancer drugs. However, resistance may also be observed in tumors with the wild-type p53 gene, since this gene is not the only regulator of apoptosis (12).

In head and neck cancers, high p53 mutation rates have been associated with tobacco consumption and worse prognosis (8,13). Although point mutations in the p53 gene are observed in nasopharyngeal cancer, the mutation rate is lower than other tumors (14-16). On the other hand, the mutant p53 can be easily detected by its extended half-life (12). Immunohistochemical studies have shown significant p53 overexpression in NPC material (3,17).

In this study, p53 protein immunoreactivity was investigated in paraffin sections of primary nasopharyngeal tumors and metastatic cervical lymph nodes and association with clinical and histopathological characteristics was evaluated.

Materials and Methods

Patients and tissue preparation: Ninety-seven paraffin sections from 81 patients with NPC, treated from 1990 to 1996, were examined by immunohistochemistry and are correlated with clinical features and treatment outcome. Seventy-six samples were derived from primary tumors and 21 samples from metastatic lymph nodes. In twenty-nine specimens, NPC and dysplastic lesions adjacent to carcinoma were observed. Among these patients, 52 were male and 29 were female (male/female ratio, 1.8/1). The age range was between 12 and 78 years (median 47 years).

The reliability of the sections from earlier years was achieved by random sampling. Sections were taken from paraffin tissue blocks containing tumor tissue. All tissue specimens were routinely processed, formalin-fixed and paraffin-embedded. The thickness of the paraffin block sections was 4 µm.

Biopsy specimens were classified into three main groups according to the WHO classification. Eight patients were WHO type I, 3 patients were WHO type II and 70 patients were WHO type III NPCs according to the differentiation degree (18).

Clinical staging. For clinical staging, patients were evaluated by clinical examination, endoscopy of nasopharynx, nasopharyngeal and cervical computed tomography (CT), routine blood biochemistry, whole-body bone scintigraphy, abdominal ultrasonography and lung X-ray. Clinical staging of the patients was performed according to the classification system of the AJCC 4th edition criteria (19). There were 13 T1, 23 T2, 16 T3 and 29 T4 tumors. Nodal stages were 7 No, 9 N1, 46 N2 and 19 N3.

Immunohistochemistry. Sections were deparaffinized and rehydrated in distilled water. For the microwave antigen retrieval method, sections were immersed in 75 ml citrate buffer and placed in a domestic microwave oven at full power (750 watt) twice for 5 min. Microwaved sections were incubated for 20 min more in hot buffer and then placed in phosphate-buffered saline (PBS, pH 7.6). In the immunohistochemical study, anti-p53 monoclonal antibody (p53 DO7, Novocastra Laboratories, diluted in 1:50), biotin-horse radish peroxidase-labelled streptavidin amplification system (Signet catalog no 2246) and amino ethyl carbosole (AEC) chromogen (Signet catalog no 1026) were used. Primary antibody was applied for 60 min at room temperature (20). Positive control sections were included in each run and consisted of rhabdo-mysarcoma-positive for a germline p53 point mutation at exon 7, codon 248 (C-T), identified by direct genomic sequencing (21). As negative controls, nonimmunized mouse sera were used and the primary antibody was omitted.

Depending on the percentage of nuclei exhibiting a positive immunohistochemical staining reaction for the p53 protein, the samples were classified into two major groups. Samples were considered to have negative reaction if there was no evidence of staining or if staining existed in <10% of tumor cells. Samples with nuclear staining in ≥10% tumor cells were classified as having a positive immunoreaction for p53.

Statistical analysis. Statistical significance was evaluated by Chi-square tests with the criterion of p<0.05. Survival rates were calculated by the Kaplan Meier method.

Results

Strongly positive nuclear staining was observed in all neoplastic cells in the positive control test. Specimens from NPC patients showed diffuse or focal, and mild or high positive nuclear staining (Figure 1). Immunoreactivity was more pronounced in the basal layer of normal and dysplastic epithelium (Figure 2).

In 29 patients there was dysplastic mucosa present around the tumor and in 16 of them similar immunoreactivity was observed in the tumor and the neighboring dysplastic mucosa. However, p53 staining was not observed in the dysplastic or carcinomatous region in 10 cases. Two samples displayed immunoreactivity in tumor cells but no reactivity in the dysplastic mucosa. In one sample, immunoreactivity was found in dysplastic mucosal cells but not in the adjacent carcinoma.

Among a total of 97 samples, positive staining for p53 protein was observed in 83 (85.5%) samples while no staining was found in 14 (14.5%) cases. Immunoreactivity was observed in 62 (81.5%) of the primary nasopharyngeal biopsy specimens. All metastatic lymph nodes displayed significant immunoreactivity (Figure 3). The labelling index was higher than 10% in all but one of the metastatic nodes.
The correlation between p53 expression and histological type, stage, age and sex distributions was tested and are summarized in Table I. After statistical analysis according to Chi-square test and Yates’ correction, no significant difference was demonstrated ($p > 0.05$).

The median follow-up was 62.5 (range 25-116) months and the 5-year cumulative survival rate was $50.9\% \pm 6.2\%$ (SE). There was no statistically significant correlation with p53 immunoreactivity and overall and disease-free survival (Figure 4).
Table I. Patient characteristics and p53 overexpression.

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Discussion

An understanding of the underlying mechanisms of cell proliferation and death will provide a new insight into the mechanisms of neoplastic transformation and resistance to antineoplastic therapy. Molecular studies on different tumor types aim to investigate the tumor-specific characteristics explaining the underlying mechanism.

NPC is a tumor type for which radiation is the primary choice of treatment (1). However, the response of the tumor to radiation therapy cannot be estimated. Different factors such as tumor hypoxia, clonogenic regeneration of the tumor, intrinsic radioresistance and the effect of some oncogenes (raf and H-ras) may affect radioresistance and only a few of these are well defined (22). Biological parameters involved in carcinogenesis and determining the response to radiation may have a strong impact on the type and duration of radiotherapy. p53 is one of the radioreistance markers determined by in vivo and in vitro studies (22). In this study, overexpression of the p53 protein was investigated in nasopharyngeal biopsy samples.

Previous studies indicated that point mutations of the p53 gene are not frequent (10%) in NPC (7,14,15). However, p53 overexpression determined by immunohistochemistry is high. An overall immunoreactivity rate of 95% with strong immunoreactivity (>10%) in 65% of the patients has been reported in Chinese patients (23). In our material, similarly, the p53 immunostaining is 85.5% and the ≥10% immunoreactivity rate is 69%. All of the metastatic cervical lymph node specimens showed positive immunostaining for p53. Dysplastic lesions adjacent to tumor showed coexpression of p53 protein. These findings indicate that inactivation of the p53 protein may occur both during early and late stages of tumor development. Immunohistochemical, molecular and functional studies conducted simultaneously may bring insight into the mechanisms leading to p53 inactivation at various stages of transformation.

The high overexpression rate (65-85%) when considered with a mutation rate of only 10% by DNA sequence analysis suggest that mechanisms other than mutations are responsible for p53 inactivation. Enzymatic degradation and stabilization of the p53 protein by complex formation with viral proteins may also lead to inactivation (9). Inactivation of the p53 protein by complex formation with SV40 large T antigen, adenovirus E1B protein or HPV E6 protein may lead to tumor development (3,5,9,24). The E6 protein inactivates p53 by inducing its degradation by an epigenetic mechanism (7,22). Consequently, in HPV(+) cervix carcinomas and head and neck carcinomas, the half-life of p53 protein is short and hence its detection rate is low. In contrast, the suggested mechanism in NPC is not the induction of degradation but the extended half-life (which does not mean overexpression) of the protein. However, such a correlation has not been observed in a study investigating the association between EBV infection and p53 overexpression (3).

Immunohistochemical changes in the p53 protein are observed in more than half of human cancers (6). In biopsy samples of patients with basal cell hyperplasia, dysplasia and in situ carcinoma an increased immunoreactivity rate of 72-92% was found in precancerous lesions while no reactivity was detected in the normal epithelium (25). Our observation of increased reactivity in the dysplastic areas surrounding the tumor is in accordance with this finding. It has been reported that a sensitivity of 75% (36-100%) and a positive predictive value of 63% (8-100%) may be achieved by immunohistochemical studies when applied before DNA analysis (24). Thus, detection of the p53 protein by immunohistochemistry does not reflect the wild-type and mutant protein ratios.

The role of p53 in NPC has been extensively evaluated, with most reporting wild-type status, although protein overexpression is observed in 31-95% of primary biopsy specimens (14-17, 26). Amongst these multiple reports, only one study reported p53 overexpression being associated with poor survival (26). In our study, there was no statistically significant correlation with p53 immunoreactivity and overall and disease-free survival.

Although the association between NPC and p53 is not clear, our study confirms that p53 overexpression is present in a considerable subset of patients with NPC. Immunoreactivity in the surrounding dysplastic mucosa indicates that p53 overexpression may occur during early stage disease. On the other hand, a high degree of immunoreaction in metastatic lymph nodes suggests that inactivation is also present in progressed disease. The mechanisms by which this overexpression occurs warrants further studies. Cancer gene therapy has been a rapidly developing field over the past 15 years. Several studies have been investigated the therapeutic potential of p53 gene...
therapy in NPC (27-29). There are promising results in this field and it seems that gene therapy will soon develop into a powerful new therapeutic modality for NPC.

Acknowledgements

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References


