Analysis of p53 Mutations and the Expression of p53 and p21$^{WAF1/CIP1}$ Protein in 15 Cases of Sebaceous Carcinoma of the Eyelid

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Purpose. The purpose of this study was to detect mutation of the p53 gene, to assess its relationship with p53 or p21$^{WAF1/CIP1}$ expression, and to evaluate the correlation between p53 mutation or p21$^{WAF1/CIP1}$ expression and clinicopathologic findings in sebaceous carcinoma of the eyelid.

Methods. Fifteen conventional paraffin-embedded samples of sebaceous carcinoma of the eyelid were analyzed. Using the single-strand conformation polymorphism technique, the authors sequenced coding exons 5–8 of the p53 gene. The expression of p53 and p21$^{WAF1/CIP1}$ protein was analyzed by immunohistochemistry.

Results. In 10 of the 15 cases (66.7%), point mutations were detected in the p53 gene. CC to TT double-base changes (tandem mutations), which are known to be induced only by UV, were not detected in any of the mutations. Correlations between p53 mutation and expression were found to be statistically significant ($P = 0.007$). There was no significant correlation between p53 mutation and clinicopathologic findings or p21$^{WAF1/CIP1}$ expression. However, there was a significant inverse correlation between p21$^{WAF1/CIP1}$ expression and presence of lymph node metastasis ($P = 0.007$).

Conclusions. Among human cancers, sebaceous carcinoma of the eyelid may be one of those showing most frequent mutation of the p53 gene, which may not be caused by exposure to UV. p21$^{WAF1/CIP1}$ downregulation may be associated with lymph node metastasis. (*Invest Ophthalmol Vis Sci.* 2010;51:7–11) DOI:10.1167/iovs.09-4127

Sebaceous carcinoma is a rare and potentially aggressive skin tumor that occurs most often in the periorbital area, usually in the eyelid (38.7%).[1] There is an unusual abundance of sebaceous glands in the ocular region, particularly in the tarsus (meibomian glands) and in association with the cilia (Zeis glands). In the United States it is generally acknowledged that basal cell carcinoma accounts for approximately 90% of malignant eyelid tumors, sebaceous carcinoma for approximately 5%, squamous cell carcinoma for approximately 4%, and others, including melanoma, for approximately 1%.[2] On the other hand, sebaceous cell carcinoma of the eyelid is relatively common in Asia, comprising 25% to 40% of all malignant eyelid neoplasms.[3] It can be locally invasive in the eyelid and conjunctiva and can metastasize to regional lymph nodes and distant organs.[4] In terms of prognosis, the reported mortality is 6% to 30%.[5–7]

The p53 tumor suppressor gene (Fig. 1) plays a central role in the maintenance of normal cell growth and differentiation and is frequently described as the guardian of the genome. p53 prevents cells that contain damaged DNA from proliferating, either temporarily by arresting the cell cycle so that DNA repair can occur, or permanently by entering the cell into a pathway of programed cell death (apoptosis). p21 is a transcriptional target of p53 and plays a crucial role in mediating growth arrest in response to DNA damage. Alterations in these signaling pathways can allow the cell to begin neoplastic growth. A large body of data indicates that mutations in the p53 tumor suppressor gene play an important role in the multistep process of carcinogenesis. Although p53 mutation has been investigated in a variety of human malignancies, to date only a few studies have focused on the mutation status of the p53 gene in sebaceous carcinoma of the eyelid.[8,9]

In lung and breast cancer, the mutation status of the p53 gene has been reported to be a useful prognostic marker or a predictor of therapy response.[10,11] Furthermore, in squamous cell carcinoma of the eye and adnexa and basal cell carcinoma, p53 mutation analysis has implicated ultraviolet (UV) radiation as the major carcinogen involved.[12–15] Thus it is clearly desirable to evaluate the correlation between p53 mutation and clinicopathologic features of sebaceous carcinoma of the eyelid, and to assess the involvement of UV in its etiology.

Therefore, in the present study, we analyzed 15 cases of sebaceous carcinoma of the eyelid for mutations of the p53 gene and further analyzed the expression of p53 and p21 by immunohistochemistry.

Materials and Methods

Patients and Tissues

Sebaceous carcinomas of the eyelid that had been surgically resected from 15 patients at Oita University Hospital between 1991 and 2006 were reviewed. Histologic evaluation was based on conventional paraffin-embedded sections stained with hematoxylin-eosin. To confirm the diagnosis, all the cases except for one (case 14) were subjected to

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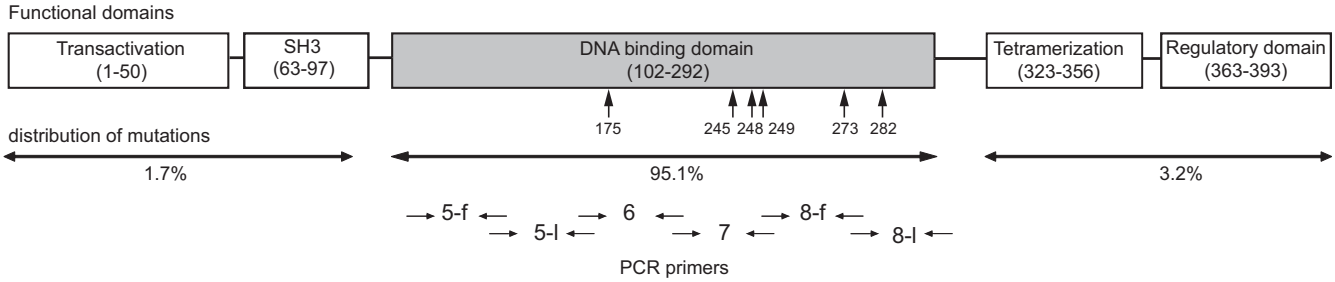

Figure 1. Schematic representation of the structure of p53 protein. The p53 protein comprises several domains. The SH3 domain contains a proline-rich region that is required for p53-mediated apoptosis. The DNA binding domain is the core domain. The *numbers* refer to the amino acid residues. Positions of hotspots are indicated under the figure (*arrows*). The great majority of p53 mutations (95.1%) affect the DNA binding domain of the p53 protein.[31] The 6 PCR primers were designed to amplify the entire region of the DNA binding domain (exons 5–8) on polymerase chain reaction.

oil-red-O histochemical staining for cytoplasmic lipid on frozen sections. The clinicopathologic data for the patients are listed in Table 1. The TNM clinical classification was based on the 2002 International Union Against Cancer (UICC) classification at the initial examination. Two patients were classified as T1, eight as T2, four as T3, and the remaining one as T4. No patient exhibited lymph node or distant organ metastasis at the initial examination. The mean follow-up time was 7.3 years (range, 1.6–17.3). After complete surgical resection, one patient was found to suffer local recurrence, five patients showed metastasis to lymph node or distant organ. Two patients (cases 2 and 8) died of metastasis. Informed consent was obtained from each participant, and the present study was approved by the Ethics Committee of Oita University Hospital (Approval number: P-06-07) and carried out in accordance with the Declaration of Helsinki principles.

Extraction of Genomic DNA from Paraffin-Embedded Carcinoma Tissues

The sections were deparaffined with xylene, rinsed with 100% (v/v) ethanol, and stained with toluidine blue. While observing carcinoma tissues by light microscopy, we directly cut out the carcinoma cells from the serial sections using sterile needles. Laser-capture microdissection was performed on some sections to separate tumor from normal tissues. The collected carcinoma cells were incubated in 0.5% SDS-TE buffer with 125 μg/mL proteinase K (Wako, Osaka, Japan) at 55°C overnight. Finally, genomic DNA was obtained by routine phenol-chloroform extraction, followed by ethanol precipitation.

Mutation Analysis of the p53 Gene

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis was performed for the four exons (exons 5–8) encoding the DNA binding domain of p53, previously defined as containing the "hot spot" regions of mutation in human cancer (Fig. 1). Subsequently, the presence of mutations was confirmed by sequencing analysis of the PCR product.

We used the same primers as those used in the previous study.[16] Each PCR reaction was performed using 100 ng of genomic DNA extracted from carcinoma cells, 0.625 unit of polymerase (Blend *Taq*; TOYOBO, Osaka, Japan), 1x buffer (TOYOBO), 0.2 mM dNTPs, and 0.2 μM forward and reverse primers in a total reaction volume of 25 μL PCR conditions were as follows: one cycle of 94°C for 3 minutes, 35

Table 1. p53 Mutation and Clinicopathologic Information

Case	Sex/Age (y)	Site	Size (mm)	Stage	Recurrence and Metastasis	p53 Mutation					
						Exon	Codon	Nucleotid*	Amino Acid Change	Mutation Effect	IHC
1	F/83	LL	5 × 10	T2	−	5	126	TACt to TAGt	Tyr to stop	Nonsense	+
2	F/71	LL	3 × 5	T1	+ (lymph node, parotid gland, brain)						−
3	M/82	UL	5 × 10	T2	−						−
4	F/59	LL	5 × 10	T2	+ (local)						−
5	M/78	UL	5 × 15	T4	+ (orbital invasion)	6	193, 215	CAT to CGT, AGT to GGT	His to Arg, Ser to Gly	Missense, missense	+
6	M/85	LL	5 × 5	T1	−	7	245	GGC to AGC	Gly to Ser	Missense	+
7	F/76	LL	8 × 8	T2	−	7	248	cCGG to cTGG	Arg to Trp	Missense	+
8	M/75	UL	30 × 15	T3	+ (lymph node, parotid gland, lung)	5	173	GTG to ATG	Val to Met	Missense	+
9	M/61	LL	17 × 17	T3	+ (lymph node)	8	272	gGTG to gATG	Val to Met	Missense	+
10	F/61	UL	8 × 10	T2	−	8	275	TGT to TAT	Cys to Tyr	Missense	+
11	F/67	LL	8 × 8	T2	−						−
12	M/68	LL	6 × 6	T2	−	int6/−1		agGT to aaGT		Splice	−
13	F/36	LL	7 × 10	T2	+ (lymph node)	5	175	CGC to CAC	Arg to His	Missense	+
14	M/73	UL	7 × 16	T3	−						−
15	F/47	UL	8 × 14	T3	+ (lymph node)	int5/+1		TGgt to TGtt		Splice	−

LL, lower eyelid; UL, upper eyelid; IHC, immunohistochemistry; +, positive; −, negative.
* Underlining shows dipyrimidine sites.

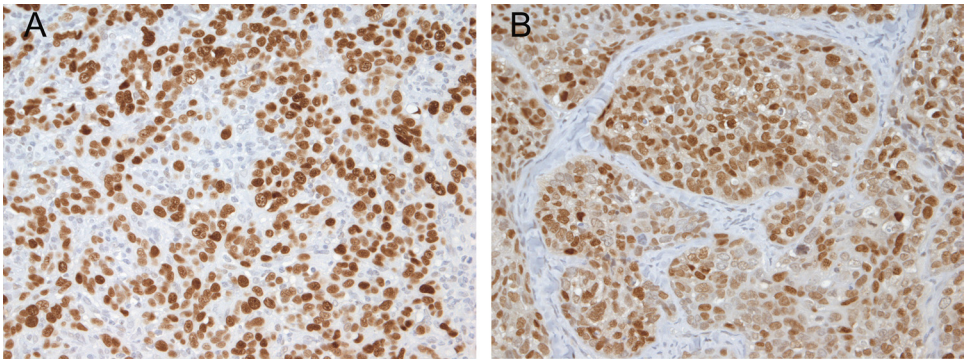

FIGURE 2. Nuclear immunoreactivity for p53 (**A**) and p21 (**B**) observed in specimens of sebaceous carcinoma of the eyelid.

cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by reaction at 72°C for 5 minutes. PCR products were loaded onto analysis gel (GeneGel Excel 12.5/24; GE Healthcare Bioscience, Uppsala, Sweden) with an electrophoresis unit (Genephor System; GE Healthcare Bioscience) at 15°C, in accordance with the manufacturer's protocol. After electrophoresis, the gels were stained with a staining kit (DNA Silver Staining kit; GE Healthcare Bioscience) and shifted bands were collected. DNA fragments recovered from the gels were re-amplified with the same primer again and then sequenced using a genetic analyzer (ABI PRISM 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA) with a sequencing kit (BigDye Terminator Cycle Sequence Ready Reaction kit; Applied Biosystems).

Immunohistochemical Detection of p53 and p21 Protein

The tissue sections were thoroughly deparaffinized and rehydrated according to the standard protocols. For antigen retrieval, the sections were immersed in 10 mM sodium citrate buffer (pH 6.0; Iatron Co., Tokyo, Japan), autoclaved at 120°C for 10 minutes, and cooled to room temperature (RT). They were then treated with 3% (v/v) H_2O_2 for 5 minutes at RT to inactivate endogenous peroxidase activity.

The slides for p53 and p21 protein detection were respectively blocked with 10% (v/v) rabbit serum (Nichirei, Tokyo, Japan) and 10% (v/v) goat serum (Nichirei) for 30 minutes at RT. Subsequently, immunohistochemistry with p53 mouse monoclonal Ab (DO-7; DAKO, Carpinteria, CA) and p21 (H-164) rabbit polyclonal Ab (Santa Cruz Biotechnology) was performed as follows. The tissue sections were incubated with the following primary antibodies: p53 and p21 diluted 1:1000 with diluting solution (DAKO) for 18 h at 4°C, respectively. The sections were then washed with 1x phosphate-buffered saline (PBS) and incubated for 30 minutes with either biotinylated rabbit anti-mouse IgG or biotinylated goat anti-rabbit IgG (Nichirei). After being washed with 1x PBS, they were incubated with a solution of avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories Inc., Burlingame, CA) for 15 minutes, according to the manufacturer's recommendations, then washed again with 1x PBS. Peroxidase activity was detected with H_2O_2/diaminobenzidine (DAB) substrate solution and the sections were counterstained with hematoxylin before dehydration and mounting.

The percentage of p21-positive nuclei scored was calculated as the percentage of positive nuclear cells in relation to the total number of tumor cells in at least three different representative high-power (×400) fields, each containing at least 100 tumor cells.

The immunoreactivity with anti-p53 Ab was generally judged as negative or positive. We also counted the percentage of positive nuclear cells in relation to the total number of tumor cells as described above.

Statistical Analysis

Statistical analyses in this study were carried out with commercial software (Stat View statistical software package; Abacus Concepts, Berkley, CA). Fisher's exact probability test was used for evaluating the association between p53 gene status and p53 immunostaining or clinical data. Mann–Whitney U test was used for evaluating the association between the percentage of p21-positive nuclei and p53 gene status or clinical data. Data are presented as the mean ± SD. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

Sequence Analysis of the p53 Gene in Sebaceous Carcinoma of the Eyelid

Tissues from 15 cases of sebaceous carcinoma of the eyelid were analyzed. In 10 (66.7%) of these cases, point mutations were detected from exons 5–8 in the p53 gene, which encode the DNA binding domain of p53 protein (Fig. 1). As shown in Table 1, of the 10 cases with p53 mutation, 9 exhibited a single point mutation, and the remaining one exhibited two point mutations in the same exon. Of the 11 mutations, 9 were localized in exons 5 ($n = 3$), 6 ($n = 2$), 7 ($n = 2$), and 8 ($n = 2$), and the remaining two were present at introns. Of the 9 exon mutations, 8 were missense mutations and the remaining one was a nonsense mutation. Of the two intron mutations, one was present at the 5′ splice site of intron 5, and the other one at the 3′ splice site of intron 6.

Of the 11 mutations, G:C to A:T, A:T to G:C, G:C to C:G and G:C to T:A mutations were detected in 7 (63.6%), 2 (18.2%), 1 (9.1%), and 1 (9.1%) cases, respectively. CC to TT tandem mutations, which were induced only by UV, were not detected in any of the mutations. And only 6 (54.5%) of the 11 mutations occurred at dipyrimidine sites, with G:C to A:T mutations (36.4%; Table 1).

p53 and p21 Immunohistochemistry

It is known that p53 protein accumulates in nuclei when the p53 gene is mutated. Therefore, we next analyzed the 15 cases by immunohistochemistry with anti-p53 monoclonal antibody (DO7 Ab). Positive immunoreactivity for p53 was detected in 8 (53.3%) of the 15 cases. As shown in Figure 2A, positive immunoreactivity was confined to the nucleus and undetectable in the cytoplasm. In 6 of the 8 cases showing p53 immunoreactivity, the proportion of positively stained tumor cells exceeded 75%, whereas in the other 2 cases, 50–75% of the cells were immunoreactive. The remaining 7 cases showed no detectable p53 immunoreactivity.

Immunohistochemistry for p21 revealed positive immunoreactivity in the nucleus in all the cases analyzed (Fig. 2B). In 5 of the 15 cases, 20–50% of tumor cells were immunostained, whereas in the remaining 10 cases, more than 50% of the tumor cells were p21-positive.

Table 2. Relationship of p53 Immunostaining to p53 Gene Status

	p53 Mutation	p53 Wild	Total
p53 IHC(+)	8	0	8
p53 IHC(−)	2	5	7
Total	10	5	15

Fisher's exact test, $P = 0.007$.

Relationship of p53 Immunostaining to p53 Gene Status

Eight of the 10 cases in which p53 mutations were detected exhibited positive immunoreactivity. Of these 8 cases, 7 harbored missense mutations and the remaining one a nonsense mutation. Furthermore, in the 2 cases that harbored mutations but were unstained by immunohistochemistry, the mutations were found to be localized at the 5′ splice site of intron 5 and the 3′ splice site of intron 6, respectively (Table 1). On the other hand, in the 5 cases without mutations, positive immunoreactivity was not detected in any of them. The correlations between p53 immunoreactivity and the presence or absence of mutations in the p53 gene were found to be statistically significant ($P = 0.007$; Table 2).

p53 Mutation and Clinicopathologic Findings

The mutation status of the p53 gene was not found to be correlated with patient sex and age, tumor site and size, or nodal status. p53 gene mutations were detected in 6 (60%) of 10 cases of sebaceous carcinoma at tumor stages T1 and T2, and in 4 (80%) of 5 cases at stages T3 and T4 (Table 1). There was no correlation between p53 mutation status and tumor stage when compared stage T1/T2 with stage T3/T4 ($P = 0.60$). There was no significant correlation between p53 mutation and the proportion of p21-positive nuclei [p53 mutation (+) 60.0 ± 12.6 vs. p53 mutation (–) 43.0 ± 21.4; $P = 0.58$].

p21 Expression and Clinicopathologic Findings

The proportion of cells positively immunostained for p21 protein was found to be correlated with neither patient sex and age nor tumor site and size. Interestingly, we found that the proportion of p21-positive nuclei was inversely correlated with the presence of lymph node metastasis [lymph node meta (–) 65.8 ± 15.2 vs. lymph node meta (+) 41.0 ± 12.9; $P = 0.007$] (Fig. 3), suggesting that p21 downregulation may be associated with lymph node metastasis.

Discussion

In our present study, we found that the p53 gene was mutated in 66.7% of sebaceous carcinomas of the eyelid. It has already been reported that the p53 gene is mutated in many carcinomas including those of the ovary (47.8%), colorectum (43.2%), lung (38.6%), stomach (32%) and breast (25.1%) (http://www-p53.iarc.fr). On the basis of these accumulated data, we suggest that sebaceous carcinoma of the eyelid is one of the carcinomas most frequently harboring mutations, and that inactivation of p53 may be important for its development.

However, the pathologic significance of p53 gene mutations in the development of cancers has not been fully elucidated. In breast and prostate cancers, mutations are rarely detectable at the early stage, and tend to become more frequently detectable at advanced stages, suggesting that p53 gene mutations might occur at an advanced stage of tumor progression.[17,18] In contrast, in gastric and ovarian cancers, mutations tend to be detectable in both advanced and early-stage tumors, suggesting that mutations are not confined to any particular stage.[19,20] In the present study, p53 gene mutations were detected in 6 (60%) of 10 cases of sebaceous carcinoma at tumor stages T1 and T2, and in 4 (80%) of 5 cases at stages T3 and T4 (Table 1). The frequency of p53 mutation was similar between low stage tumors (T1, T2) and high stage tumors (T3, T4), suggesting that mutational inactivation of p53 may be important in the development of early-stage tumors.

Sebaceous carcinoma has been classified as a skin tumor from a clinical as well as histopathologic standpoint. However, we found that p53 mutations in sebaceous carcinoma of the eyelid differed from those of skin tumors in some respects. It has been reported that both the locations of mutations in the p53 gene and the type of base substitutions involved differ between skin tumors and internal malignancies.[21,22] Exposure to UV radiation is well known to play a causative role in skin carcinogenesis,[22] and tends to cause tandem mutations, especially CC to TT mutations, in the p53 gene.[21,23,24] Indeed, tandem mutations are common (14%) in skin tumors but very rare in internal malignancies (0.8%).[22] In the present study, tandem mutations were not detected in any of the 11 mutations we encountered. Furthermore, UV exposure also tends to produce mutations at dipyrimidine sites.[21,23,24] Although 92% of mutations in skin tumors occur at dipyrimidine sites, mutations at dipyrimidine sites have been reported to account for 61% of mutations in internal malignancies.[22] In the present study, only 6 (54.5%) of the 11 mutations were found at dipyrimidine sites. On the other hand, in one case (case 13), mutation at codon 175 was detected. Although mutations at codon 175 of p53 are frequently found in internal malignancies, they have not yet been found in skin tumors.[12] Thus, in sebaceous carcinoma of the eyelid, it is clear that the locations of the mutations and the types of base substitutions involved differ from those of skin tumors, and in fact resemble those characterizing internal malignancies. On the basis of these data, we speculate that UV exposure may not be responsible for carcinogenesis in sebaceous carcinoma of the eyelid.

It has been believed that in carcinoma cells with p53 mutation, the mutant p53 protein tends to become stabilized and accumulates in the nucleus. Therefore, immunohistochemistry for p53 protein has been used as a convenient tool for detection of p53 gene mutation status. In the present study, eight of the 10 cases in which p53 mutations were detected exhibited positive immunoreactivity. These 8 cases harbored mutations within exons. Furthermore, in the 2 cases that harbored mutations but were unstained by immunohistochemistry, the mutations were found to

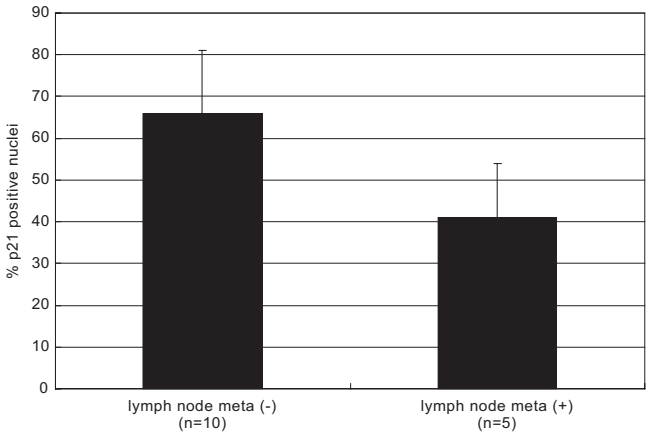

Figure 3. The proportion of p21-positive nuclei was inversely correlated with the presence of lymph node metastasis (lymph node meta (–) [$n = 10$], 65.8 ± 15.2 vs. lymph node meta (+) [$n = 5$], 41.0 ± 12.9; $P = 0.007$). Lymph node meta (–), tumors without lymph node metastasis; lymph node meta (+), tumors with lymph node metastasis.

be localized at the intron splice site. On the other hand, in the 5 cases without mutations, positive immunoreactivity was not detected in any of them. These findings suggest that p53 protein had accumulated in the nucleus in the cases harboring p53 gene mutations within exons, but not in the cases harboring mutations at the intron splice site. In the present series, the correlations between immunohistochemical data and sequence data were statistically significant ($P = 0.007$; Table 2). Although there were two false negative cases in which the p53 gene mutation was present at the intron splice site, it is suggested that this immunohistochemical approach with anti-p53 monoclonal antibody (DO7 Ab) is a useful and convenient approach for clarifying the p53 mutation status of sebaceous carcinoma of the eyelid.

p21 is an inhibitor of cyclin-dependent kinases, induced by p53-dependent and p53-independent pathways, which can block progression through the cell cycle.[25] In the present series, p21 expression was not correlated with p53 mutation status. Consistent with our findings, Ito et al.[26] also reported that p21 expression was not correlated with p53 mutation in patients with endometrial carcinoma. Furthermore, DiGiuseppe et al.[27] reported that p21 expression might be induced by a p53-independent pathway in human pancreatic carcinomas. On the basis of these findings, we speculate that p53-independent pathways may be important for p21 expression in sebaceous carcinoma of the eyelid.

It is believed that sebaceous carcinoma of the eyelid metastasizes typically to regional lymph nodes, and that involvement of preauricular or cervical lymph nodes is associated with a 5-year mortality rate of 50%–67%.[1] Therefore, it is important to predict the risk factors for lymph node metastasis. Interestingly, we found that positive immunoreactivity for p21 was inversely correlated with the presence of lymph node metastasis, suggesting that p21 downregulation may be associated with lymph node metastasis in sebaceous carcinoma of the eyelid ($P = 0.007$; Fig. 3). Indeed, it has been reported that in colon cancer and bladder cancer, the amount of p21 staining is inversely correlated with disease stage and lymph node metastasis.[28,29] Jiang et al.[30] also reported that low expression of p21 related to high probability of lymph node metastasis in breast carcinoma. Therefore, we suggest that p21 immunoreactivity may be used as a tool for prediction of nodal metastasis in sebaceous carcinoma of the eyelid.

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