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Altered patterns of the interferon-inducible gene IFI16 expression in head and neck squamous cell carcinoma: immunohistochemical study including correlation with retinoblastoma protein, human papillomavirus infection and proliferation index

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Aims: To investigate whether the expression of interferon (IFN)-inducible gene IFI16 is inversely related to proliferative activity *in vivo*, we compared immunohistochemical reactivity of IFI16 in a series of head and neck squamous cell carcinomas (HNSCCs) with their proliferation index and the cell cycle regulator pRb. As human papillomavirus (HPV) infection is manifested by changes in the function or expression level of host genes such as IFN-inducible genes, we also investigated the presence of HPV DNA to determine whether head and neck cancers associated with HPV DNA can be distinguished from tumours that are presumably transformed by other mechanisms.

Methods: Thirty-six HNSCCs were evaluated for IFI16, pRb and Ki67 expression by immunohistochemistry. The presence of HPV was also detected by polymerase chain reaction. Nine tumours were located in the oropharynx (tonsillar area) and 27 in the larynx.

Results: HPV DNA was found in 14 of 25 (56%) laryngeal SCCs and in five of nine (56%) tonsillar SCC specimens examined; 17 out of the 19 HPV-DNA-positive cases showed high-grade IFI16 expression. Overall, proliferative activity was significantly related to tumour differentiation and histological grading. IFI16 protein expression was significantly inversely correlated with Ki67 ($P = 0.039$). Low-proliferating tumours positive for IFI16 staining showed a marked expression of pRb and a better prognosis than those whose tumours had low IFI16, pRb levels and a high proliferation index.

Conclusions: To our knowledge, this is the first expression analysis of the IFN-inducible IFI16 gene in HNSCC. Low-proliferating tumours positive for IFI16 staining showed a marked expression of pRb and a better prognosis than those whose tumours had low IFI16, pRb levels and a high proliferation index.

Keywords: HNSCC, HPV, IFI16, interferon

Abbreviations: AIM, absent in melanoma; HIN, haematopoietic interferon-inducible nuclear protein; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; IFN, interferon; MNDA, myeloid nuclear differentiation antigen; PCR, polymerase chain reaction

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Introduction

Human malignancies arising in the head and neck region are a significant cause of morbidity and mortality worldwide.[1] Their epidemiology has been well described, whereas the molecular steps involved in their pathogenesis are poorly understood. Mucosotropic high-risk papillomaviruses (HPVs) known to cause cervical and anogenital cancers have been proposed to play a role in the aetiology of some head and neck squamous cell carcinomas (HNSCCs).[2–5] The presence of high-risk HPV DNA in a subgroup of HNSCCs supports this hypothesis.[6]

Molecular studies have provided important data on the role and oncogenic mechanisms of high-risk HPV in carcinogenesis. By expression of the viral oncoproteins E6 and E7, the virus dysregulates crucial cellular mechanisms, such as the cell cycle and the apoptotic pathway, by inhibiting the activity of the cellular p53 and Rb tumour suppressor proteins, respectively.[7,8] Moreover, some *in vitro* studies have demonstrated the ability of HPV oncoproteins to control signalling pathways that lead to the expression of interferon (IFN) and IFN-inducible genes.[9,10] It has been reported that HPV16 E6 binds to the carboxyl-terminal domain of transcription factor IRF3 and inactivates its transactivating function.[11] Expression of HPV16 E6 in human keratinocytes diminishes the induction of IFN-β gene expression by Sendai virus and consequently the expression of IFN-inducible genes. By using microarray analysis Chang and Laimins have identified IFN-inducible genes and STAT-1 as major targets of HPV31.[12,13] Lastly, Nees *et al.* have used the same technique to demonstrate that HPV16 E6 in cervical keratinocytes decreases the expression of IFN-α and -β, down-regulates nuclear STAT-1 protein, and decreases its binding to the IFN-stimulated response element.[14]

The IFN-inducible genes comprise members of the HIN-200 (haematopoietic interferon-inducible nuclear proteins with a 200-amino-acid repeat) gene family found on human and mouse chromosome 1.[15,16] This family consists of a number of highly homologous human and murine proteins with similar primary amino acid sequences and biological characteristics. The mouse HIN-200 family includes Ifi202, Ifi204, Ifi203 and D3, while the human family includes IFI16, MNDA (myeloid nuclear differentiation antigen) and AIM2 (absent in melanoma 2). A highly conserved 200-amino-acid domain present singly or in duplicate is a structural motif found in all members which harbours an LXCXE motif that is a potential site for binding to the retinoblastoma gene product.[17] Over-expression of the 204 protein, encoded by the mouse Ifi204 member, in mouse embryo fibroblasts (MEF) retarded their proliferation, delayed G_1 progression into S-phase and led to an accumulation of cells with a DNA content equivalent to cells arrested in late G_1.[18,19] These effects on cell cycle progression were strictly dependent on the association of p204 with the Rb protein.[20,21] Moreover, over-expression of the p204dm inactivated by mutation at both LXCXE motifs increases cell proliferation and leads to malignant transformation capability, such as growth under low-serum conditions and formation of tumours in athymic nude mice.[22] Altogether these results suggest a strict correlation between Ifi204 expression and inhibition of cell growth.

All HIN-200 family members are expressed in haematopoietic cells, and some molecules show a tightly regulated expression pattern in certain cell types.[23,24] Recent findings from our and other laboratories in normal adult human tissues have shown that IFI16 is also expressed in epithelial and endothelial cells.[25,26] Prominent IFI16 expression is seen in stratified squamous epithelia, particularly intense in basal cells in the proliferating compartments, whereas it gradually decreases in the suprabasal more differentiated compartment. In the cervix, only the squamous epithelium of the exocervix was positive, whereas the glandular mucosa of the endocervix was negative. Expression of IFI16 in the most basal, rapidly cycling cells appears counter to the proposed function of HIN-200 proteins in negatively regulating cell growth. This expression pattern in squamous epithelia is therefore somewhat similar to that seen in the haematopoietic system, with IFI16 being highly expressed in progenitor stem cells and some differentiated cell types (i.e. lymphocytes, monocytes), yet not expressed in granulocytes and macrophages.[23] These results therefore indicate that mere expression of IFI16 may not be sufficient to inhibit cell growth. Its selective expression in some cell lineages, high level in basal squamous epithelial cells, and loss of expression in many cells following or during differentiation indicate that it may be involved in regulating cell differentiation or proliferation. Consistent with a role for p200-family proteins in cell growth regulation, there are indications that viral oncoproteins functionally inactivate p202.[27] Expression of AIM2 is lost by frame-shift mutations in colorectal tumours, and loss of MNDA expression in prostate carcinoma is linked to progression to more aggressive metastatic prostate cancer.[28,29] Recent studies have revealed that increased levels of IFI16 in prostate epithelial cells contribute to senescence-associated irreversible cell growth arrest. Moreover, its over-expression in human prostate cancer cell lines,

that did not express IFI16, inhibited colony formation.[30] Altogether, these observations support the idea that the loss of function of p200-family proteins, by providing growth advantage to the affected cells, may contribute to the development of cancer.

To define whether IFI16 expression reflects a genetic change in multistep cancer transformation, we analysed the relationship between its expression, tumour proliferation, grade of differentiation, and expression of a well-known tumour suppressor (pRb) in HNSCC.

In the present study, we demonstrate the correlation between IFI16 expression, HPV status, and low proliferation index in a series of HNSCCs and discuss its biological relevance.

Materials and methods

PATIENTS AND TUMOUR SPECIMENS

Patients with a diagnosis of SCC of the head and neck region and treated at the Maggiore Hospital, Novara, were identified through the surgical pathology files and tumour registry. Clinical information was collected by chart review. The clinical staging and identification of the anatomical site of the tumours were based on the International Union Against Cancer (1987) TNM classification of malignant tumours.[31] Thirty-six patients who underwent surgical treatment for SCC of the upper aerodigestive tract were included. The distribution of the tumours by anatomical site was as follows: 27 tumours in the larynx, of which 12 were assigned as supraglottic and 15 as glottic, and nine in the tonsil. The age of the patients ranged from 45 to 87 years (mean 63 years). Thirty-one patients were male and five were female. The follow-up of the patients ranged from 9 to 98 months (mean 37.5). The diagnosis and assessment of the grade of differentiation were made according to Wiernik *et al.*[32] on haematoxylin and eosin-stained sections from 10% buffered formalin-fixed paraffin-embedded tissues.

IMMUNOHISTOCHEMISTRY

Immunohistochemical analysis was performed on sections from the same paraffin-embedded tissue placed on silane-coated glass slides. In brief, sections were deparaffinized, and the endogenous peroxidase activity was blocked with 0.3% H_2O_2 in 1 × PBS. For antigen retrieval, the slides were placed in a Coplin jar filled with a 10 mM trisodium citrate solution at pH 6.0, and heated for 26 min at 750 W and then for 15 min at 300 W in a conventional pressure cooker underwent cycle in a microwave oven. Slides were incubated in blocking solution (5% donor serum plus 0.2% Triton X100 in PBS) to prevent non-specific binding and then with an optimal dilution of the primary antibody (1 : 5000 for the rabbit polyclonal anti-carboxy-terminal IFI16).[25] After washing in 1 × PBS, they were incubated with the appropriate biotinylated secondary antibody, followed by streptavidin–horseradish peroxidase complex (Immunotech, Marseille, France). Immunostaining was performed by incubation of the slides in diaminobenzidine solution (DAB) (Roche, Mannheim, Germany); the enzymatic reaction was monitored under a conventional microscope. Finally, slides were counterstained with haematoxylin for 30 s, dehydrated, and mounted with Eukitt (Bioptica, Milan, Italy). In all experiments, a routine control was included in which the primary antibody was omitted. The substitution of the antibody with preimmune serum was the internal control. IFI16 immunostaining was confined to the nuclei in both neoplastic and non-neoplastic cells. In all peritumoral normal epithelia, only the nuclei of the basal and parabasal cells were positive. In positive tumours, more than 50% of neoplastic cells expressed IFI16. As expected, IFI16 was detected in the nuclei of reactive fibroblasts, endothelial cells, and some inflammatory cells surrounding the cancer tissue. These non-neoplastic cells were always stained, and this served as an internal positive control when lesions were judged negative.

The immunoreactivity for pRb was assessed using the Rb (C-15) rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Slides were incubated with the antibody at 1 : 1000 dilution for 1 h at room temperature and processed as described above. Immunohistochemical staining for the proliferative fraction was performed with the Ki67/MIB-1 mouse monoclonal antibody (Immunotech) at 1 : 1000 dilution as described. The percentage of positive cells was assessed in each case and the staining was divided into two categories: < or >20% of Ki67+ cells, as reported in Table 1.

All slides were reviewed and scored in a blind test by two pathologists (M.P. and G.V.).

DNA EXTRACTION

A modified version of the Wright and Manos method (*PCR protocols: a guide to methods and applications*. New York: Academic Press, 1990) was used for DNA extraction.[33] One to three 10-μm sections were taken from the paraffin block, placed in a 1.5-ml plastic tube containing 150 μl of digestion buffer (50 mM Tris–HCl, 1 mM EDTA, 0.5% Tween 20) and 0.2 mg/ml proteinase K (Roche). Samples were incubated for 4 h at

Table 1. Summary of primary site, histopathological grading, and IFI16, pRb, Ki67 expression in 36 head and neck squamous cell carcinomas

Case	Site	Grading	IFI16	Ki67	pRb
1	TA	Low	3*	<20%	Pos
2	TA	High	1	>20%	Pos
3	TA	High	3	>20%	Pos
4	TA	High	2	>20%	Pos
5	TA	Low	2	>20%	Pos
6	TA	High	1	>20%	Neg
7	TA	High	2	>20%	Pos
8	TA	High	2	>20%	Pos
9	TA	High	2	>20%	Neg
10	GL	High	0	>20%	Neg
11	GL	Low	3	<20%	Pos
12	GL	High	2	>20%	Pos
13	GL	High	0	>20%	Neg
14	GL	Low	2	<20%	Pos
15	GL	Low	3	>20%	Pos
16	GL	Low	2	<20%	Pos
17	GL	Low	3	<20%	Pos
18	GL	Low	2	<20%	Pos
19	GL	Low	3	<20%	Pos
20	GL	Low	3	<20%	Pos
21	GL	High	0	<20%	Pos
22	GL	High	2	>20%	Pos
23	GL	Low	2	>20%	Pos
24	GL	Low	1	>20%	Neg
25	SG	Low	3	<20%	Pos
26	SG	Low	3	<20%	Pos
27	SG	High	0	>20%	Neg
28	SG	Low	3	<20%	Pos
29	SG	Low	3	<20%	Pos
30	SG	Low	1	>20%	Pos
31	SG	Low	2	<20%	Pos
32	SG	High	1	>20%	Neg
33	SG	High	1	>20%	Pos
34	SG	High	2	>20%	Neg
35	SG	Low	1	<20%	Pos
36	SG	High	1	>20%	Pos

*IFI16 staining: 0, null; 1, weak; 2, nuclear; 3, nuclear and/or nucleolar.
TA, Tonsillar area; GL, glottic; SG, supraglottic.

65°C. Protease was then destroyed by a 5-min incubation at 95°C and the samples were immediately centrifuged for 5 min at 11 000 ***g***.

The concentration of the extracted DNA was spectrophotometrically evaluated at 260 nm.

POLYMERASE CHAIN REACTION

Sample DNA (100–300 ng) from each extraction were used for polymerase chain reaction (PCR) on a DNA thermal cycler (Touch Down, HYBAID). To control the integrity and the suitability of the isolated DNA and/or the presence of PCR-inhibitory substances, each specimen was confirmed by successful amplification of a fragment of the β-globin gene using the primers previously described, which resulted in an amplified product of 260 bp.[34]

Two oligonucleotide primers, homologous to the highly conserved L1 region of the HPV genome, were used to assess the presence of the HPV genome.[3] The sequence was as follows: GP5+ (sense), 5′-TTT GTTACTGTGGTAGATACTAC-3′ and GP6+ (antisense), 5′-GAAAAATAAACTGTAAATCATATTC-3′. The amplified product was of 150 bp. All the primers used were synthesized in an oligonucleotide synthesizer (PRIMM). To detect the specific HPV subtype, the HPV+ specimens were sequenced using the GP5+ primer. Amplification reactions were set up in a final volume of 100 μl containing: 100–300 ng of the extracted sample, 1 × Taq buffer [20 mM Tris–HCl (pH 8.4); 50 mM KCl, 2.5 mM $MgCl_2$], 200 μM of each of the four dNTPs, 100 pm of each of the primers; 2 U Taq DNA Polymerase (Gibco, BRL, Carlsbad, CA, USA). After an initial 'hot start' of 10 min at 85°C, DNA amplification was performed for 40 cycles, using the following thermocycle-step parameters: 94°C for 1 min to denature the DNA, 2 min at 55°C for annealing, and

90 s at 72°C with a final extension of 10 min at 72°C. The negative controls were samples with water replacing target DNA in the reaction mixture. Twenty microlitres of the reaction mixture were analysed by electrophoresis on 1 or 2% agarose (for β-globin and L1 amplification, respectively) and the reaction products were visualized by ethidium bromide staining. To avoid contamination of specimens with viral DNA, standard precautions concerning spatial separation of pre- and post-PCR steps, aliquoting of reagents and single use of scalpels for processing tissue specimens were strictly followed. A set of negative controls (water, unrelated genomic DNA) was included during all steps of the DNA isolation and amplification procedure. None was positive for HPV DNA.

STATISTICAL ANALYSIS

The significance of the correlation between the values was estimated by a χ^2 test or Fisher's exact test for categorical data.

All patients had a 2-year minimum follow-up. The date of diagnosis was considered the starting day of observation; patients who died of other causes without evidence of disease or who were unavailable for follow-up were either censored at the time of death or at last follow-up. The overall survival curves were calculated according to the Kaplan–Meier method. The log rank test was used to test for differences between subgroups.

All *P*-values were considered statistically significant if ≤ 0.05. All data were processed with GraphPad Prism statistical software (San Diego, CA, USA).

Results

IMMUNOHISTOCHEMICAL STUDIES

The morphological features that best characterized HNSCCs were related to keratinization and included the presence of intracellular bridges, extracellular deposition of keratinaceous amorphous material, cytoplasmic keratinization, and the formation of keratin pearls. The tumours were therefore divided into well keratinized (low grade) and poorly keratinized (high grade). The former were characterized by variably sized nests of squamous cells with abundant highly keratinized cytoplasm and numerous central keratin pearls. The central portions of the nests often contained large concentric keratin whorls. Individual tumour cells had distinct borders, abundant homogeneous cytoplasm with a clear, glassy appearance, and numerous easily identified cytoplasmic bridges. At the periphery of the tumour, this histological type tended to have an infiltrative pattern with small groups, cords or bands of tumour cells advancing into the adjacent stroma. In contrast, the poorly keratinized tumours were composed of a relatively monomorphic population of small ovoid to spindled cells with a high nuclear to cytoplasmic ratio arranged in variably sized sheets, nests, and cords. Individual cells often had indistinct cell borders, relatively small amounts of cytoplasm with minimal or no squamous maturation and resembled immature basal cells; keratin pearls and cytoplasmic bridges were very rare. Tumour necrosis was mostly seen in the central portions of the nests. By using these two categories we divided our series of cases into 19 low-grade and 17 high-grade carcinomas.

Two patterns of IFI16 intracellular distribution were observed, as illustrated in Figure 1: (i) strong nucleolar staining with weak or negative nuclear expression (Figure 1A); (ii) diffuse or granular nuclear staining (Figure 1B). By contrast, immunoreactivity for IFI16 was not detected in other neoplastic lesions (Figure 1C). Overall, in low-grade carcinomas, tumour nests displayed a peculiar IFI16 distribution: the central keratinized cells were mostly negative, as shown in Figure 2, and tended to be sharply demarcated from the more peripheral, often single layer of positive basal cells resembling Figure 1A. The advancing front of the tumour into the adjacent stroma tended to be pushing or expansile rather than infiltrating and most of its cells were positive. In high-grade tumours, IFI16 immunoreactivity mostly resembled Figure 1B, showing weak nuclear granular staining. IFI16 immunostaining was assessed semiquantitatively according to both the proportion of positively stained cells and the IFI16-staining pattern as follows: grade 0 (no staining); grade 1 (<50% of positive cells with weak nuclear staining resembling Figure 1B); grade 2 (>50% of positive cells with strong nuclear staining); grade 3 (>50% of positive cells with a prominent nucleolar staining resembling Figure 1A). The difference between grades 2 and 3 relates to the IFI16 nucleolar localization and more uniform staining in the nucleoplasm which resembles its normal expression pattern as previously reported.[25] In Table 1, the specimens are categorized according to the primary tumour site, clinical data, and histological features and all the IFI16 immunoreactivity results are reported. Nine of 17 high-grade tumours were IFI16– or weakly positive, scored as grade 0 and 1. By contrast, 16 of 19 low-grade tumours were IFI16+, scored as grade 2 and 3. There was a statistical relationship between grading and IFI16 expression; grades 2 and 3 IFI16 expression were related to low-grade carcinoma ($P = 0.032$). This correlation was higher when only nucleolar (grade 3)

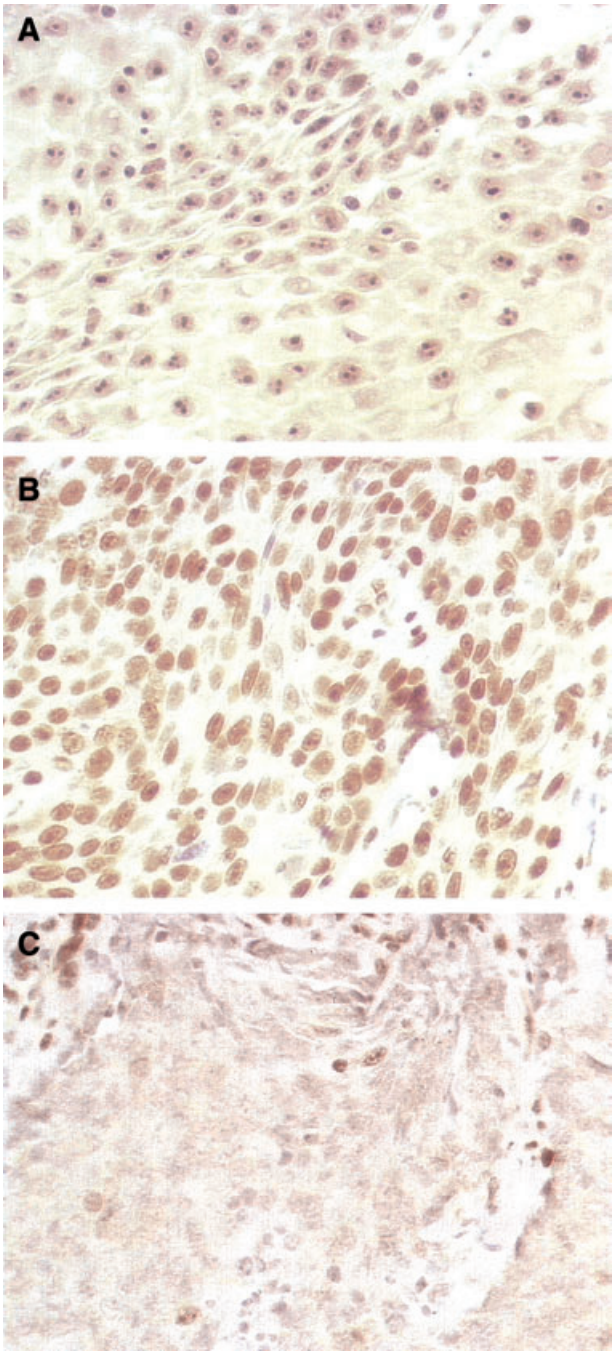

Figure 1. Examples of immunostaining patterns for the IFI16 protein. **A,** Strong nucleolar staining with weak or negative nuclear expression. **B,** Diffuse and granular nuclear staining. **C,** Negative staining. Immunohistochemical staining shows IFI16-positive and -negative cells in brown (DAB substrate) and counterstained with haematoxylin, respectively.

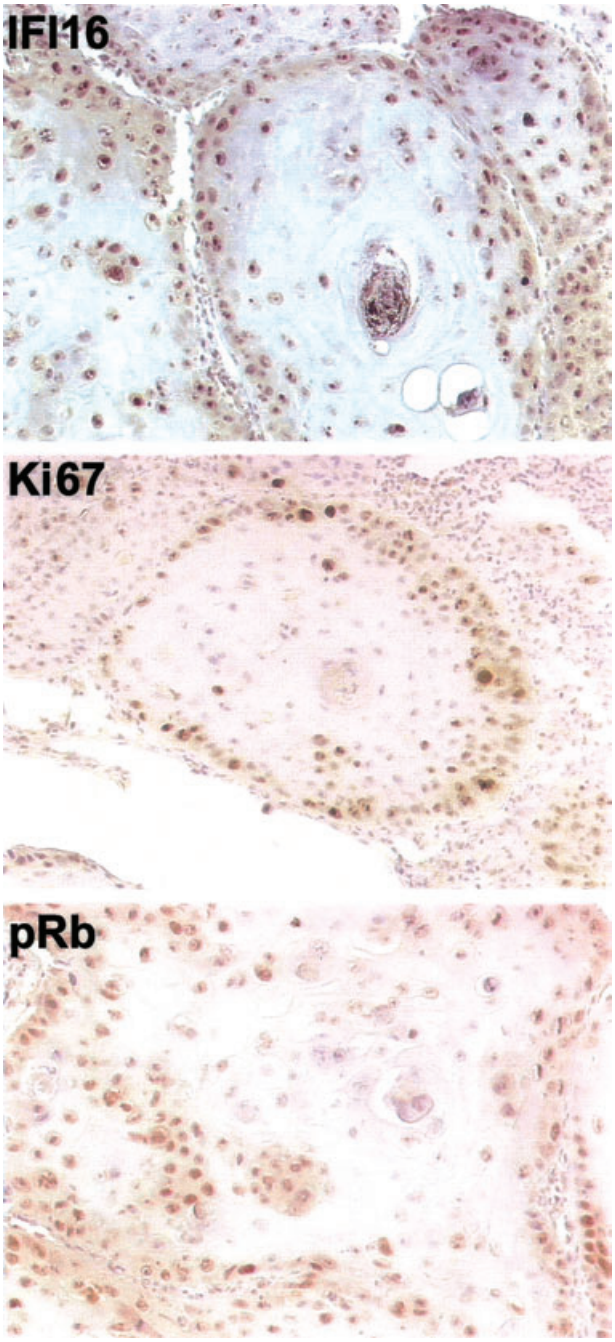

Figure 2. Representative photomicrographs of the immunostaining patterns of IFI16, Ki67, and pRb in a case of low-grade HPV+ squamous cell carcinoma of the larynx. Immunohistochemical staining shows positive and negative cells in brown (DAB substrate) and counterstained with haematoxylin, respectively.

IFI16 expression was considered ($P = 0.0034$). No correlation existed between IFI16 expression and tumour site, patient age or gender.

Serial sections were also analysed for their proliferative activity by immunostaining for Ki67. In normal epithelia Ki67+ cells were mostly located in the basal cell layer, in the tumour mass; they were randomly

distributed in all levels of the epithelium and nearly all cells of the invading front were positive. In low-grade squamous cell carcinomas, positive Ki67 staining was detected only in cells at the periphery of the tumour cell nests (Figure 2). The central keratinizing areas and adjacent tumour cells were negative. Conversely, the staining was more diffuse in high-grade carcinomas (Figure 3). Overall, the proliferative activity, as defined by Ki67 immunostaining, was significantly related to tumour differentiation and histological diagnosis; 16 of 17 high-grade tumours displayed >20% Ki67+ cells as reported in Table 1. Ten of these highly proliferating lesions showed negative or weak nuclear IFI16 staining. By contrast, 13 of 19 low proliferating lesions strongly expressed IFI16 and displayed grades 2 and 3. Our results indicate that intense nuclear and/or nucleolar IFI16 staining strongly correlates with a lower proliferation rate ($P = 0.039$).

Since it has been demonstrated that HIN-200 proteins exert their antiproliferative activity through the pRb pathway,[21] we processed sections by immunohistochemistry for the expression of the Rb protein to correlate IFI16 expression with pRb status. In normal squamous epithelium, the parabasal cells were pRb+ and the basal cells showed no detectable pRb. The normal mucosa adjacent to the carcinoma stained positive in all cases. The pattern of Rb staining tended to be diffuse with variable intensity of nuclear staining. Positive nuclei were randomly distributed throughout the tumour with a higher number of positive cells in low-grade as opposed to high-grade tumours. Tumours with more than 30% of nuclei staining were interpreted as positive. Staining for pRb was detected in 28 cases. As expected, in the eight negative cases the normal squamous epithelium from the surrounding area showed a normal pRb expression pattern. The surrounding dysplasia stained positive for both pRb and IFI16 proteins, indicating that loss of expression was dependent on the stage of progression (Figure 3). Six of these eight Rb– tumours showed IFI16 staining grade 0 or 1, and all of them displayed a high proliferation rate as determined by Ki67 staining, and illustrated in Figure 3. By contrast, the majority of pRb+ tumours also showed marked expression of IFI16 (grades 2 and 3, see Figure 2). On dividing IFI16 staining negative (grades 0 and 1) versus positive (grades 2 and 3), a significant correlation between IFI16 and Rb expression was observed ($P = 0.009$). In the multivariate analysis, IFI16 and Rb expression retained their significant association with low grade of proliferation ($P = 0.014$). Although the correlation was not absolute, IFI16 nucleolar staining as shown in Figure 1A and Figure 2 was more likely to occur in lesions with strong Rb expression.

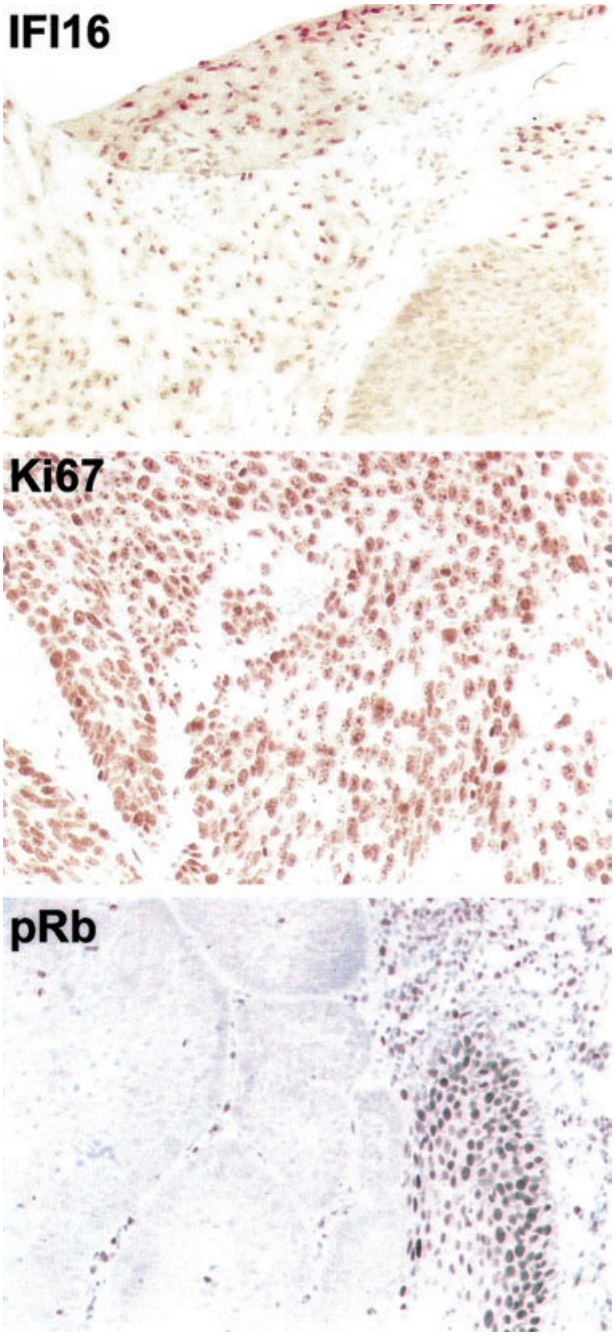

Figure 3. Representative photomicrographs of the immunostaining patterns of IFI16, Ki67, and pRb in a case of high-grade HPV– squamous cell carcinoma of the larynx. Immunohistochemical staining shows positive and negative cells in brown (DAB substrate) and counterstained with haematoxylin, respectively.

Distribution of IFI16 and pRb staining in the high and low proliferating tumours is summarized in Figure 4.

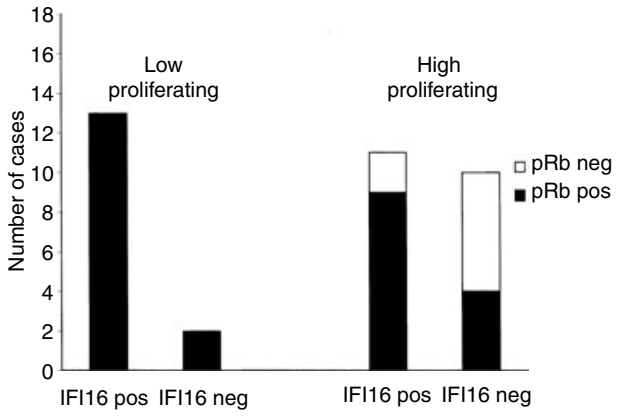

Figure 4. Relationship between proliferation index, pRb immunostaining and IFI16 expression levels scored as positive (grades 2 and 3) and negative (grade 0 and 1) in 36 head and neck squamous cell carcinomas.

OCCURRENCE OF HPV SEQUENCES AND CLINICOPATHOLOGICAL PARAMETERS

In two specimens (from patients with only one specimen available), β-globin DNA could not be amplified, probably due to disintegrated DNA rather than PCR inhibition, since PCR products were produced after adding control HPV DNA. Subsequently, all HPV-DNA-positive carcinomas were sequenced using the GP5+ primer to determine the specific HPV subtype. Table 2 shows the results obtained by PCR analysis together with the IFI16 expression pattern and the clinicopathological characteristics of the patients. HPV DNA was found in 14 of 25 (56%) laryngeal SCC and in five of nine (56%) tonsillar SCC specimens examined. In the HPV+ specimens, the high-risk HPV types 16 and 18 were predominantly represented. HPV-16 DNA was detected in 14 cases (41%), and HPV-18 was detected in five (15%). For simplicity, in Table 2 IFI16 immunohistochemical staining is reported as positive (including patterns 2 and 3 from Table 1) and negative (including patterns 0 and 1 from Table 1). HPV+ and IFI16+ cases were predominantly low grade, and 17 out of 19 HPV-DNA-positive cases showed high levels of IFI16 expression with a statistically significant correlation ($P = 0.0035$). Of interest is that, among laryngeal tumours, the correlation between the presence of HPV DNA and sustained IFI16 expression was higher ($P < 0.0001$). Figure 5 illustrates the distribution of IFI16 expression in relation to HPV status.

Clinicopathological parameters analysed included age, gender, primary site, tumour extension, nodal status, clinical stage, tumour grade differentiation, local control, and follow-up. Follow-up information was available in all cases. Twelve patients died of disease during the follow-up period, ranging between 9 and 33 months after the date of diagnosis. Of note, only one of them showed the highest grade of IFI16 expression. Twenty patients were alive at the end of the follow-up. The overall survival curves by the Kaplan–Meier method (Figure 6) showed a better prognosis for patients whose tumours had the highest grade of IFI16 expression ($P = 0.034$). Since grade 3 reflects its cellular localization in normal cells where it exerts its physiological action, one can hypothesize that in these tumours IFI16 can still regulate epithelial cell growth. This finding is in line with the highest correlation observed between grade 3 IFI16 expression and low-grade carcinomas. By contrast, no association was observed between HPV+ or HPV– tumours and patient demographics or disease status. Comparison of HPV+ and HPV– subgroups showed no significant differences in overall survival ($P = 0.239$).

Discussion

A hallmark of HNSCC is its extensive clinical heterogeneity.[35] This makes it difficult to assess its malignancy and predict the outcome of treatment. Molecular markers defining certain genotypes and phenotypes and representing tumour subgroups with more homogeneous behaviour must thus be found. Tumours associated with oncogenic HPV may represent such a subgroup.[36,37]

Although some of IFI16's biochemical properties and molecular interactions have been delineated *in vitro*, its physiological role and *in vivo* expression in pathological situations such as tumours have not been investigated.[38,39] This study is the first systematic investigation of IFI16 expression in a series of primary HNSCCs according to their histopathological grading and proliferation index. We have demonstrated a strong correlation between IFI16 expression and histopathological grade: sustained IFI16 immunoreactivity was uniformly observed in low-grade and was barely detectable or negative in high-grade HNSCC. Interestingly, most HPV+ cases expressed high IFI16 levels.

The retinoblastoma (Rb) gene product is constitutively expressed in the nucleus of every normal cell.[40] Alterations in the gene that result in loss of the Rb protein or in production of an abnormal protein product have been found in a wide variety of epithelial tumours, including breast carcinoma, bladder cancer, and non-small-cell lung cancer.[41,42] Previous studies have demonstrated that pRb immunoreactivity in HNSCC is enhanced in well-keratinized as opposed to poorly differentiated tumours.[43,44] It has also recently

Table 2. Clinicopathological parameters, IFI16 and HPV detection in 36 head and neck squamous cell carcinomas

Case	Age	Sex	Primary site*	T†	N‡	Stage	Grade	IFI16§	HPV present/type	Local control	Follow-up, months
1	47	M	TA	3	2b	IV	Low	Pos	Neg	Yes	DID 37¶
2	70	M	TA	2	2b	IV	High	Neg	16	Yes	DODM 22**
3	49	M	TA	2	1	III	High	Pos	Neg	Yes	NED 58††
4	60	F	TA	1	2	IV	High	Pos	Neg	Yes	NED 25
5	48	M	TA	2	0	II	Low	Pos	Neg	Yes	NED 44
6	73	M	TA	2	2	IV	High	Neg	18	Yes	DODM 15
7	56	M	TA	1	0	I	High	Pos	16	No	DOLD 18‡‡
8	61	M	TA	2	1	III	High	Pos	18	Yes	NED 25
9	56	F	TA	2	0	II	High	Pos	16	Yes	NED 24
10	63	M	GL	2	2	IV	High	Neg	Neg	Yes	DODM 14
11	45	M	GL	1a	0	I	Low	Pos	16	Yes	NED 30
12	59	M	GL	3	2b	IV	High	Pos	16	Yes	DODM 20
13	69	M	GL	2	0	II	High	Neg	Neg	Yes	NED 21
14	59	M	GL	1a	0	II	Low	Pos	16	Yes	NED 78
15	57	M	GL	2	0	II	Low	Pos	16	No	DOLD 21
16	73	M	GL	1a	0	I	Low	Pos	ND§§	Yes	NED 84
17	70	M	GL	1	0	I	Low	Pos	16	Yes	NED 82
18	70	M	GL	3	2b	IV	Low	Pos	16	Yes	DODM 33
19	74	M	GL	3	0	III	Low	Pos	16	Yes	NED 42
20	72	M	GL	1a	0	I	Low	Pos	16	Yes	DID 40
21	60	F	GL	3	2	IV	High	Neg	ND	No	DOLD 20
22	69	M	GL	3	0	III	High	Pos	18	Yes	NED 80
23	87	F	GL	1b	0	I	Low	Pos	16	Yes	NED 98
24	64	M	GL	4	0	IV	Low	Neg	Neg	No	DOLD 11
25	59	M	SG	4	2c	IV	Low	Pos	Neg	Yes	DID 30
26	59	M	SG	1	0	I	Low	Pos	Neg	Yes	NED 42
27	58	M	SG	1	0	I	High	Neg	Neg	Yes	NED 40
28	61	M	SG	1	0	I	Low	Pos	16	Yes	NED 70
29	67	M	SG	3	0	III	Low	Pos	16	Yes	NED 36
30	69	M	SG	2	2c	IV	Low	Neg	Neg	Yes	NED 48
31	75	M	SG	4	1b	IV	Low	Pos	18	No	DOLD 9

Table 2. (*Continued*)

Case	Age	Sex	Primary site*	T†	N‡	Stage	Grade	IFI16§	HPV present/type	Local control	Follow-up, months
32	55	M	SG	2	0	II	High	Neg	Neg	Yes	DID 13
33	71	F	SG	2	0	II	High	Neg	Neg	Yes	NED 48
34	55	M	SG	3	1	IV	High	Pos	18	No	DOLD 10
35	58	M	SG	3	0	III	Low	Neg	Neg	Yes	NED 32
36	61	M	SG	2	2	IV	High	Neg	Neg	Yes	DODM 30

*TA, Tonsillar area; GL, glottic; SG, supraglottic.
†Tumour not more than 20 mm in greatest dimension (T1); tumour more than 20 mm but not more than 40 mm (T2); tumour more than 40 mm (T3); tumour invades adjacent structures (T4).
‡No regional lymph node metastasis (N0); single ipsilateral lymph node metastasis, 30 mm or less in greatest dimension (N1); single ipsilateral lymph node metastasis, more than 30 mm but not more than 60 mm in greatest dimension (N2a); metastasis in multiple ipsilateral lymph nodes, none more than 60 mm in greatest dimension (N2b); metastasis in bilateral or contralateral lymph nodes, none more than 60 mm in greatest dimension (N2c); metastasis in a lymph node more than 60 mm in greatest dimension (N3).
§IFI16 staining: Neg, grades 0 and 1; Pos, grades 2 and 3.
¶Died of an intercurrent disease.
**Died of distant metastasis with local control.
††No evidence of disease at the indicated months.
‡‡Died of the local disease.
§§Not determined.

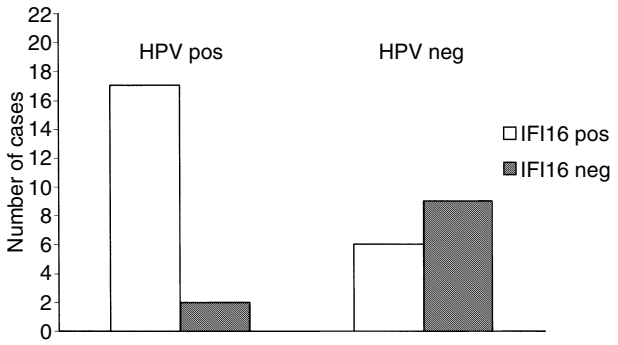

Figure 5. Relationship between HPV status and IFI16 expression levels scored as positive (grades 2 and 3) and negative (grade 0 and 1) in 36 head and neck squamous cell carcinomas.

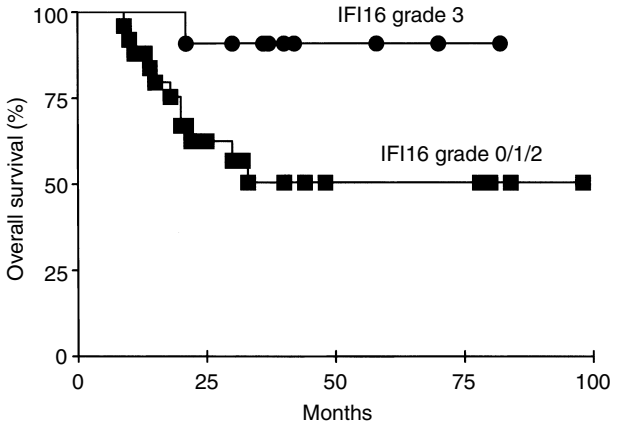

Figure 6. Cumulative prognostic value of IFI16 expression in 36 head and neck squamous cell carcinomas expressed as probability of overall survival.

been reported that pRb levels in HPV E7- expressing cells are low due to degradation through the ubiquitin–proteasome pathway.[45–47] Our results, too, indicate that pRb immunostaining occurs mainly in low-grade tumours. In addition, we observed that pRb significantly associates with IFI16 expression and is not affected by the presence of the HPV DNA, as shown by the similar immunoreactivity score of the HPV+ and HPV– groups ($P = 0.417$). By contrast, 17 out of 19 HPV-DNA-positive cases showed high-grade IFI16 expression, a statistically significant observation ($P = 0.0035$) consistent with Laimins *et al.*'s demonstration that IFN-inducible genes are major targets of HPV infection.[12,13] Although the presence of HPV in

HNSCC can easily be overestimated if assessed by PCR only, as in this study, and further investigations are needed to establish a causal association between HPV infection and IFI16 expression in HNSCC, we may assume that IFI16 up-regulation in HPV+ tumours is a predictive marker of a low proliferation rate.

During the cell cycle, the active underphosphorylated pRb is only present in G_0/G_1 phase and acts as a negative regulator of its progression.[48] The transcriptional activator E2F/DP family is preferentially bound to the hypophosphorylated Rb protein and results in the functional inactivation of E2F, thereby blocking its growth-promoting activity mainly during G_0/G_1.[49] During transition to the S-phase, pRb is inactivated upon phosphorylation by cyclin-dependent-kinases (cdks), causing a substantial decrease in E2F/pRb binding.[50] This allows E2F to function as a transcriptional activator on target promoters and express certain genes containing E2F binding sites at the beginning of S-phase proceeding to uncontrolled cell growth.

In the mouse system overexpression of Ifi204, the homologue of the IFI16 gene, retarded cell proliferation, delayed G_1 progression into S-phase and accumulated cells with a DNA content equivalent to cells arrested in G_1.[18,19] These effects were strictly dependent on the association of progression with Rb, suggesting that Ifi204 protein controls the cell cycle through the pRb system.[21] Importantly, Xin *et al*. have reported that ectopic expression of IFI16 in prostate cancer cell lines results in colony formation inhibition that differs with respect to expression of functional pRb; the maximum inhibition was seen in LNCaP (these cells express functional Rb) and minimum inhibition was seen in DU-145 cells (these cells do not express functional Rb).[27] Consistent with these observations, we have shown that IFI16 and pRb immunostaining overlaps in those low-growth tumours and conversely are both barely detectable in more aggressive tumours. The strong association between simultaneous expression or alteration of IFI16 and pRb has some interesting implications. The first is that *in vivo* immunohistochemical analysis of HNSCC reinforces the previous results showing a close association of the mouse Ifi204 protein with the pRb system.[21] The second implication is that sustained IFI16 expression correlates with a lower proliferation rate and a higher differentiation grade, and may thus be of assistance in prognosis. The third is that, since these proteins are considered as negative regulators of the cascade of events involved in cell growth, their simultaneous down-regulation suggests a definite and irreparable alteration of mechanisms responsible for cell proliferation. In this context, we analysed the clinical relevance of IFI16 expression and found a significant correlation between a high score for IFI16 expression and overall patient survival ($P = 0.034$).

A high proliferation rate has been correlated with aggressive behaviour in tumours of various sites. In HNSCC there are data which indicate that cell proliferation indices are reliable and reproducible indicators of aggressiveness.[51,52] Consistent with these observations, in this study we report that IFI16 expression correlates inversely with proliferative activity, evaluated as the percentage of Ki67+ tumour cells. The lower proliferation rate of strongly IFI16+ tumours may be related to the antiproliferative activity of the HIN-200 proteins. Clearly, both human and mouse HIN-200 proteins can regulate gene transcription and cell growth. By contrast, it is still unclear whether their cell cycle regulation is a direct consequence of transcription regulation, i.e. direct binding to transcription factors such as NF-κB, AP-1, or E2F/DP-1, or induced indirectly by their binding to cell regulatory proteins, such as pRb.[15–17] In support of the latter hypothesis, we found that the majority of squamous cell carcinomas demonstrated a significant correlation between IFI16 and pRb expression, suggesting that, as observed in the mouse, cell cycle regulation by IFI16 may partly depend on its interaction with the pRb pathways.

Our data strongly suggest the the IFN-inducible gene product IFI16 plays a role in the growth regulation of HNSCC. The finding of high IFI16 levels in low-grade tumours together with its reported antiproliferative activity supports this hypothesis. Although the role of IFI16 in human HNSCC is unknown, the significant correlation between its levels, pRb immunoreactivity, low histopathological grading, low proliferation index, and HPV presence suggests that IFI16 expression may identify a subset of less aggressive tumours. In this regard, we found that the highest grade of IFI16 expression appeared to be a good variable in predicting overall survival in HNSCC. In summary, our data, together with the recent observations by Xin *et al*. demonstrating that IFI16 contributes to cellular senescence and irreversible growth arrest of prostate epithelial cells, suggest that loss of IFI16 function may provide a proliferation advantage to cells by bypassing cell cycle check points.[30] Further work is in progress to determine whether expression of IFI16 function in head and neck squamous epithelial cells could serve as a molecular marker to follow the severity of squamous hyperplasia and intraepithelial neoplasia in patients and, more importantly, to determine whether the loss of IFI16 function could serve as a reliable marker for development of HNSCC at an early stage.

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