Expression of retinoblastoma gene product in respiratory epithelium and sinonasal neoplasms: relationship with p16 and cyclin D1 expression

M.J. Schwerer¹, A. Sailer¹, K. Kraft¹, K. Baczako² and H. Maier³

¹Department of Pathology, Military Hospital Ulm, Ulm/Donau, Germany, ²Institute of Pathology, Ulm/Donau, Germany and ³Department of Otorhinolaryngology, Head and Neck Surgery, Military Hospital Ulm, Ulm/Donau, Germany

Summary. Transition from G1 to S phase of the cell cycle is mediated by interactions between the Retinoblastoma gene product (pRb), p16, and cyclin D1. To determine the expression of these proteins in the sinonasal mucosa immunohistochemistry was carried out on archived tissue sections from 46 patients (37 men, 9 women, age range 17 to 82 years, median 55 years). Nuclear immunostaining for these proteins was assessed and the expression rates (percentages of immunoreactive nuclei) in normal respiratory epithelium, inverted sinonasal papillomas, cylindrical (oncocytic) sinonasal papillomas, and squamous cell carcinomas were compared.

Normal respiratory epithelium showed significantly higher pRb expression in surface cells compared to basal cells (p<0.05). In contrast, abundant pRb expression in surface and basal cells was detected in columnar differentiation in sinonasal papillomas and adjacent mucosa. Cuboidal and squamous metaplasia in inverted papillomas showed significantly reduced pRb expression in surface cells compared to columnar epithelium in inverted papillomas (p<0.05, respectively). Expression of p16 was detected in all epithelial cell layers of normal respiratory epithelium, sinonasal papillomas, and adjacent mucosa. Cuboidal and squamous metaplasia in inverted papillomas showed increased p16 expression in surface cells compared to columnar epithelium in inverted papillomas (p<0.05 between squamous metaplasia and columnar epithelium). Sinonasal squamous cell carcinomas showed the coexpression of pRb and p16. Expression rates of cyclin D1 higher than 10% were detected only in invasive carcinomas but not in normal respiratory epithelium. Cuboidal and squamous metaplasia in inverted papillomas involves downregulation of pRb expression along with increased p16 expression in surface cells. Sinonasal squamous cell carcinomas coexpress pRb and p16. Overexpression of cyclin D1 in sinonasal lesions is confined to invasive squamous cell carcinomas.

Key words: Respiratory epithelium, Sinonasal lesions, pRb, P16, Cyclin D1

Introduction

The mucosal surface of the sinonasal tract is characterised by a pseudostratified respiratory epithelium. This involves columnar ciliated surface cells, mucin-producing goblet cells as well as small, round to ovoid basal cells (Balogh, 1997). Inverted papillomas and cylindrical cell papillomas represent benign neoplastic lesions which demonstrate an expansive downward proliferation of sinonasal epithelium (Michaels and Young, 1995). The epithelium in inverted papillomas undergoes a stepwise metaplastic process along with disease progression: ciliated columnar epithelium is replaced by cuboidal epithelium and finally squamous metaplasia (Michaels and Young, 1995; Schwerer et al., 2001). In contrast, cylindrical cell papillomas are characterised by a unique histological appearance with tall, slender, columnar surface cells. Microcysts filled with mucous or nuclear debris are abundantly present within the epithelium (Michaels and Young, 1995). The association between sinonasal papillomas and squamous cell carcinomas which was already reported by Hapman and Billroth in 1883 is now commonly accepted. Larger studies identified malignant progression in up to 25% of inverted papillomas (Lawson et al., 1995; Jardine et al., 2000; Klimk et al., 2000; Thorp et al., 2001). In contrast to inverted papillomas and cylindrical cell papillomas the exophytic
Dysfunction of several regulatory pathways in the cell cycle have recently been identified in sinonasal carcinoma (Califano et al., 2000). However, the role of the Retinoblastoma pathway of cell cycle control has yet not been investigated in sinonasal lesions. The Retinoblastoma gene product (pRb) is widely expressed in human tissues. Underphosphorylated pRb serves as a negative regulator by prohibiting progression from mid-G1 to S-phase. Phosphorylation of pRb on serine and threonine residues, a process which is triggered by cyclin D1/Cyclin-dependent kinase (CDK)4 and/or CDK6 complexes leads to the dissociation of transcription factors including proteins of the E2F family. Consequently, E2F stimulate the expression of genes required for S-phase control including c-myc, N-myc, and dihydrofolate reductase. The p16 protein product of the CDKN2/MTS1/INK4 tumour suppressor gene located on chromosome band 9p21 binds to and inhibits CDK4- and CDK6-mediated phosphorylation of pRb by releasing cyclin D1 from its association with CDK4/CDK6, subsequently targeting cyclin D1 for destruction. Feedback autoregulatory loops have been identified in which activated as well as inactivated pRb modulates p16 and Cyclin D1 expression (Goodrich et al., 1991; Müller et al., 1994; Lukas et al., 1995; Yeager et al., 1995; Fang et al., 1998). Dysregulation of pRb-dependent cell cycle control is associated with unrestricted proliferation and carcinogenesis in a plethora of human tissues including oral and laryngeal squamous cell carcinomas (Bartkova et al., 1995; Pavelic et al., 1996; Pande et al., 1998; Ambrosch et al., 2001; Papadimitrakopoulou et al., 2001; Akervall et al., 2002).

In the present study the expression of pRb, p16, and cyclin D1 was investigated in the respiratory mucosa. Compared to other techniques immunohistochemistry allows the phenotypic localisation of pRb, p16, and cyclin D1 expression at cellular level. Significant concordance between the immunostaining results for the Retinoblastoma pathway proteins and the findings from Western blotting has recently been demonstrated (El-Naggar et al., 1999). Hence, we applied immunohistochemistry to investigate differences in pRb, p16, and cyclin D1 expression between respiratory epithelium and sinonasal squamous cell carcinomas. Further, the expression of these proteins in inverted papillomas and cylindrical cell papillomas which represent sinonasal papillomas with an increased malignant potential was studied.

**Material and methods**

**Tissue specimens and standard histology**

Paraffin-embedded tissue specimens from 46 patients were retrieved from the archives of the Department of Pathology, Military Hospital Ulm, Ulm/Donau, Germany. The patient population comprised 37 males (80.4%) and 9 females (19.6%). Patients were 17 to 82 years of age, with a median of 55 years. Four serial slides, 5μm thick, were cut and one of them was stained with Haematoxylin and eosin (H&E). Histological examination for adequacy of lesional tissue and classification of specimens was carried out using the H&E-stained slides. The remaining three slides were used for immunohistochemistry.

**Study groups**

Normal respiratory epithelium was studied using seven cases with either normal histology or minimal chronic inflammation. Specimens showing features of more than minimal chronic inflammation, including dense lymphocytic infiltration, lymphofollicular hyperplasia, basal membrane thickening, and/or submucosal fibrosis, were excluded from this group. Further, specimens with signs of active inflammation, including neutrophilic infiltration, edema, and/or hemorrhage as well as samples with concomitant sinonasal papillomas or squamous cell carcinomas, were excluded. According to the definitions for lesions of the upper aerodigestive tract given by the World Health Organization, inverted papillomas and cylindrical cell papillomas were classified into two respective groups (Shanmugaratman and Sobin, 1991). Twenty-three cases of inverted papillomas and eight specimens of cylindrical cell papillomas were evaluated. Cases associated with concomitant sinonasal squamous cell carcinomas were excluded from these groups. Within inverted papillomas, epithelial components with columnar differentiation were present in nineteen cases. Cuboidal epithelium was present in twenty-one cases, and squamous epithelium in fourteen cases. These distinct histological components were evaluated in respective subgroups. In seventeen cases of inverted papillomas and cylindrical cell papillomas, adjacent, non-papillomatus respiratory epithelium was available and studied in a respective group. Eight cases of sinonasal squamous cell carcinomas were evaluated. Among them were three cases with carcinoma in situ and five cases with invasive carcinomas. Classification of specimens was carried out by two independent observers (M.J.S. and K.B.). Differences between their individual reports were resolved by re-examination and consensus.

**Immunohistochemistry**

The streptavidin-biotin-peroxidase technique for immunohistochemistry was applied as previously reported (Schwerer et al., 2001). Briefly, sections were dewaxed in xylol and through graded alcohols and then immersed in a citrate buffer solution (0.01 M sodium citrate, pH 6.0). Sections were boiled in a microwave oven at 500 W (six times, 5 minutes each) for antigen
Assessment of immunohistochemistry

Evaluation and statistics

Expression of pRb in respiratory epithelium and sinonasal papillomas

Expression of p16 in respiratory epithelium and sinonasal papillomas
Table 1. Expression rates (in %) of Retinoblastoma and p16 gene products in respiratory mucosa and sinonasal papillomas.

<table>
<thead>
<tr>
<th>RETINOBLASTOMA GENE PRODUCT (pRb)</th>
<th>p16</th>
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<tbody>
<tr>
<td></td>
<td>surface</td>
</tr>
<tr>
<td>Normal respiratory epithelium (n=7, all specimens negative for cyclin D1):</td>
<td>74.3</td>
</tr>
<tr>
<td>Respiratory epithelium adjacent to papillomas (total: n=17, cyclin D-1 positive: n=8):</td>
<td>86.5</td>
</tr>
<tr>
<td>Cyclin D 1 pos.</td>
<td>79.8</td>
</tr>
<tr>
<td>Cyclin D 1 neg.</td>
<td>95.7</td>
</tr>
<tr>
<td>Cylindrical cell papillomas (total: n=8, cyclin D 1 positive: n=6):</td>
<td>95.4</td>
</tr>
<tr>
<td>Cyclin D 1 pos.</td>
<td>95.4</td>
</tr>
<tr>
<td>Cyclin D 1 neg.</td>
<td>90.9</td>
</tr>
<tr>
<td>Inverted papillomas, columnar epithelium (total: n=19, cyclin D 1 positive: n=13):</td>
<td>86.1</td>
</tr>
<tr>
<td>Cyclin D 1 pos.</td>
<td>85.4</td>
</tr>
<tr>
<td>Cyclin D 1 neg.</td>
<td>92.1</td>
</tr>
<tr>
<td>Inverted papillomas, cuboidal epithelium (total: n=21, cyclin D 1 positive: n=15):</td>
<td>12.7</td>
</tr>
<tr>
<td>Cyclin D 1 pos.</td>
<td>14.6</td>
</tr>
<tr>
<td>Cyclin D 1 neg.</td>
<td>11.8</td>
</tr>
<tr>
<td>Inverted papillomas, squamous epithelium (total: n=14, cyclin D 1 positive: n=10):</td>
<td>25.7</td>
</tr>
<tr>
<td>Cyclin D 1 pos.</td>
<td>24</td>
</tr>
<tr>
<td>Cyclin D 1 neg.</td>
<td>28</td>
</tr>
</tbody>
</table>

*pos.*: positive; *neg.*: negative.

Fig. 1. Normal respiratory epithelium from a 19-year-old man. **a.** Nuclear expression of Retinoblastoma gene product (pRb) in this specimen is strongly restricted to a subset of surface cells. **b.** Nuclear expression of p16 involves the majority of basal cells along with a subset of surface cells. Serial slides from the same biopsy. x 200

Fig. 2. Non-papillomatous nasal mucosa adjacent to an inverted papilloma in a 43-year-old man. Nuclear immunoreactivity for pRb (**a**) and p16 (**b**) is present in basal as well as surface cells of the epithelium. Serial slides from the same tissue section. x 200
were detected from columnar to cuboidal epithelium and finally to squamous metaplasia (Fig. 4b.d). The difference in nuclear p16 expression in surface cells of squamous epithelium compared to columnar epithelium in inverted papillomas was significant \( p<0.05 \). Staining results are demonstrated in Table 1.

**Fig. 3.** Cylindrical cell papilloma from a 56-year-old man. Nuclear expression of pRb (a) and p16 (b) involves the majority of cells in all epithelial cell layers. Serial slides. x 125

**Fig. 4.** Inverted papilloma from a 38-year-old man. Nuclear expression of pRb (a) and p16 (b) in columnar epithelium (serial slides). Note immunopositivity for both proteins in all epithelial cell layers. Squamous epithelium from the same specimen immunostained for pRb (c) and p16 (d). Serial slides. Note lower expression of pRb in the nuclei of surface cells compared to basal cells in squamous epithelium (c). Increased nuclear immunoreactivity for p16 is present in surface cells of squamous epithelium (d) compared to columnar epithelium (b). a,b, x 100; c, d, x 200
Expression of pRb and p16 in sinonasal squamous cell carcinomas

All sinonasal squamous cell carcinomas showed nuclear immunoreactivity for pRb as well as p16 (Figs. 5a,b). The expression rates of both proteins were highly variable in both squamous cell carcinoma in situ as well as in invasive squamous cell carcinomas. Staining results are presented in Table 2.

Expression of cyclin D1 in respiratory epithelium and sinonasal lesions

Normal respiratory epithelium was constantly negative for cyclin D1 expression. As shown in Table 1 nuclear immunoreactivity for cyclin D1 was present in a subset of cases of respiratory epithelium adjacent to sinonasal papillomas, cylindrical cell papillomas, and inverted papillomas. In all cases immunoreactivity was restricted to the nuclei of basal and parabasal cells and comprised less than 10% of cells (data not shown). No differences in pRb and p16 expression were found between cyclin D1-positive versus negative specimens. As shown in Table 2 squamous cell carcinomas in situ demonstrated either the absence of cyclin D1 or minimal expression rates of this protein. Four out of five invasive squamous cell carcinomas showed nuclear cyclin D1 expression in more than 10% of tumour cells (Fig. 5c).

Discussion

Surface cells of normal respiratory epithelium showed significantly higher expression rates of pRb compared to basal cells. The basal cells represent the regenerative compartment of the sinonasal epithelium. Epithelial turnover involves dividing precursor cells which undergo terminal differentiation along with their integration into the surface cell layer (Balogh, 1997; Guichard et al., 1998). In resting cells pRb prevents the G1 to S-phase transition of the cell cycle (Goodrich et al., 1991; Fang et al., 1998). Hence, our findings indicate an association between pRb expression and terminal differentiation in columnar surface cells. Similar concepts have been proclaimed for other stratified epithelia including human oral and laryngeal squamous epithelium (Pavelic et al. 1996; Pande et al., 1998; El-Naggar et al., 1999; Ambrosch et al., 2001). In addition,

![Image of Fig. 5: Invasive squamous cell carcinoma from a 62-year-old man. Note coexpression of pRb (a) and p16 (b). Immunostaining for both proteins as well as the expression of cyclin D1 (c) predominantly involve the nuclei of tumour cells on the invasion front. Serial slides from the same tissue section. x 40](image-url)
comparable observations have been reported from animal models involving fetal mouse tissues (Szekely et al., 1992).

Sinonasal papillomas and adjacent respiratory epithelium were characterised by abundant pRb expression in all epithelial cell layers. Expression of pRb in proliferating cells in those lesions must be concluded. Changes in pRb function result from either deletion or mutation of the Retinoblastoma (Rb) gene on chromosome band 13q14.1-13q14.2, blocking of pRb by viral oncoproteins, or phosphorylation of pRb which is mediated by complexes comprised of a D-type cyclin and CDK4 and/or CDK6 (Greger et al., 1990; Goodrich et al., 1991; Dowdy et al., 1993; Pavelic et al., 1996; Caputi et al., 1998). Homozygous deletion of the Rb gene results in negative immunostaining for pRb whereas immunopositivity for pRb strongly correlates with functional Rb (Geradts et al., 1995). Hence, functional Rb in our specimens must be hypothesised. Recent studies revealed that oncoproteins of DNA viruses, including the E7 protein of human papillomavirus (HPV), form complexes with pRb involving the pocket region of pRb and a LXCXE motif of the partner protein resulting in dysregulation of pRb-mediated G1-checkpoint control (Dowdy et al., 1993). Interaction with the viral oncoprotein releases the pRb-imposed block on the activity of the positive transcription factors E2F. As a result the expression of several genes required for S-phase control, including c-myc, N-myc, and dihydrofolate reductase, is initiated (Nevins, 1992; Dowdy et al., 1993; Lee and Cho, 2002). An E7-dependent maintenance of cell proliferation conductive for viral replication is established (Nguyen et al., 2002). In addition, E7-mediated dysregulation of E2F proteins independently from pRb are currently proclaimed (Hwang et al., 2002). The development and progression of sinonasal inverted papillomas is strongly associated with HPV type 6/11 and 16/18 infection (Rady et al., 1998; Buchwald et al., 2001; Kraft et al., 2001). The presence of papillomavirus oncoprotein E7 in sinonasal papillomas has recently been demonstrated (Harris et al., 1998). However, reliable detection of E7 protein E7 requires fresh tissue and thus was not carried out in our study on paraffin-embedded specimens. Further studies are necessary to clarify the relationship between oncoprotein E7 and pRb-mediated cell cycle control in sinonasal lesions. Inactivation of the growth suppressive function of pRb can be mediated through cyclin D-CDK4/CDK6 complexes leading to phosphorylation of pRb (Goodrich et al., 1991). The CDK-inhibitor p16 encoded by the CDKN2/MTS1/INK4 tumour suppressor gene negatively regulates the activity of these complexes thus preventing uncontrolled proliferation (Lukas et al., 1995; Fang et al., 1998). Loss of functional p16 by genomic or epigenetic alterations is associated with carcinogenesis in a variety of human tumours (Esteller et al., 2002), including carcinomas in the upper and lower airways (Kratzke et al., 1996; Caputi et al., 1998; Ambrosch et al., 2001; Baba et al., 2001), the oesophagus (Roncalli et al., 1998; Mathew et al., 2001; Wong et al., 2001), the bladder (Benedict et al., 1999) as well as melanomas (Maelandsmo et al., 1996) and neuroblastomas (Omura-Minamisawa et al., 2001). In our study an abundant expression of p16 was revealed in normal respiratory epithelium as well as in cylindrical cell papillomas, columnar epithelium in inverted papillomas, and adjacent mucosa. Alterations of the CDKN2 gene result in a loss of p16 immunoreactivity (Benedict et al., 1999; El Naggar et al., 1999; Ambrosch et al., 2001). Hence, integrity of the CDKN2 gene and its transcription can be hypothesised from our immunohistochemical findings. However, additional molecular genetic studies are required to determine the status of the CDKN2 gene in sinonasal lesions. In surface cells of inverted papillomas, significantly reduced pRb expression correlated with significantly increased p16 expression along with the stepwise metaplastic process from columnar respiratory epithelium to cuboidal epithelium and finally squamous metaplasia. Decreased transcription of the Rb gene due to p16 expression has repeatedly been evidenced (Lukas et al., 1995; Yeager et al., 1995; Fang et al., 1998; Ambrosch et al., 2001). As a result, reciprocal expression patterns of pRb and p16 are evident in immunohistochemistry (Pande et al., 1998; El Naggar et al., 1999). In sinonasal squamous cell carcinomas, however, we constantly observed the coexpression of pRb and p16. Dysbalances in the reciprocal expression of pRb and p16 in sinonasal carcinomas must be proclaimed. In invasive squamous cell carcinomas, overexpression of cyclin D1 was frequently observed. The cyclin D1 protein encoded by the Cyclin D1/PRAD1/BCL-1/CCND1 gene on chromosome band 11q13 is expressed at the highest level in the middle and late G1 phase of the cell cycle (Motokura et al., 1991; Bartkova et al., 1994; Müller et al., 1994). Overexpression of cyclin D1 resulting from cyclin D1 gene amplification or rearrangement is associated with loss of pRb-mediated growth suppression on the G1 checkpoint of the cell cycle in several malignancies; for instance, in head and neck carcinomas (Bartkova et al., 1995; Papdimitrakopoulou et al., 2001; Akerval et al., 2002), lung cancer (Caputi et al., 1999; Jin et al., 2001), carcinomas of the upper and lower gastrointestinal tract (Roncalli et al., 1998; Jung et al., 2001; Nagasawa et al., 2001), the breast (Barbaresechi et al., 1997; Hielsen et al., 1997), and the female genital tract (Rolfe et al., 2001). In accordance with previous studies, minimal levels of cyclin D1 immunopositivity indicate physiological expression (Bartkova et al., 1994). Overexpression of cyclin D1 can be assumed in specimens with more than 10% of immunoreactive cells (Roncalli et al., 1998). Carcinoma in situ as well as sinonasal papillomas and adjacent respiratory epithelium showed cyclin D1 expression only in a subset of cases and expression rates lower than 10% of cells were constantly observed. In all groups of benign sinonasal lesions no differences in pRb and p16 expression were found between cyclin D1-
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