Abstract: Background. p27BBP is a regulator of ribosome assembly and an essential nuclear and cytoplasmic component of eukaryotes.

Methods. We investigated the immunochemical distribution of p27BBP in head and neck carcinomas, in the associated normal mucosa, and in regional lymph nodes.

Results. p27BBP is detectable in mucosal cells but is overexpressed in carcinomas, highly concentrated in large polymorphous nucleoli, and even larger and more evident in lymph node metastatic foci. Western blotting confirms increased p27BBP in carcinomas versus normal mucosa and also in metastatic versus normal lymph nodes. The overexpression of p27BBP corresponds to mRNA upregulation in carcinomas. Unexpectedly, a 52-kDa band specifically reacting with antibodies to p27BBP was observed in several carcinomas.

Conclusions. p27BBP alterations are common events in the transition to malignancy and are probably involved in squamous cell carcinoma progression. Immune reagents raised to p27BBP may provide additional diagnostic tools for surgical pathology of tumor boundaries and lymph nodes. The 52-kDa band may represent an abnormal form of p27BBP expressed by transformed airway epithelia.

Keywords: ribosome biogenesis; integrin β4; immunohistochemistry; head and neck carcinoma; p27BBP

Molecular markers involved in crucial steps of malignant progression may provide new diagnostic and therapeutic perspectives in different cancer types. One potential marker is an intriguing protein, p27BBP, that has already been reported to be highly increased in colorectal carcinoma and related to its progression.1

Previous research has established that this protein, originally identified as an interactor of the large cytodomain of integrin β42 and as a putative eukaryotic initiation factor (eIF6),3 is an essential regulator of the 60S ribosome assembly.4 Its evolutionary conservation (more than 85% identity between human and yeast) suggests that p27BBP...
controls a crucial event within the protein synthesis pathway that has been maintained throughout eukaryotic evolution. The molecular details of the machinery controlling the function of p27BBP are not known.

The cellular topography of p27BBP is multiple: on one side, it is found at the cytoplasmic aspect of the plasma membrane of most cells in accord with its interaction with adhesion mechanisms; on the other side, p27BBP is highly concentrated in nucleoli, witnessing its major role in ribosome biogenesis. However, p27BBP has also been found in a cytoplasmic soluble form and in a cytoskeleton-associated insoluble form. These multiple locations suggest that p27BBP may play different roles in different cellular structures but also suggest that a potential unifying role as a local controller of ribosome assembly may be served by p27BBP wherever protein synthesis occurs.

The original detection of p27BBP in epithelial cells that express the integrin heterodimer α6β4 indeed suggests a link between cell adhesion and protein synthesis regulation. In carcinomas, the role of the integrin complex α6β4 has been correlated to invasiveness and malignancy of tumor cells in carcinomas, including those of the head and neck. In this article, we attempt to uncover a correlation between the level of expression of p27BBP and its mRNA in a group of head and neck squamous cell carcinomas obtained as surgical resection samples. The biological properties of p27BBP expression have been studied in tumor samples and compared with normal epithelium samples from the same patient; in a sizable number of cases, the metastatic effusions identified in regional lymph nodes have also been studied.

One aim of this article is to propose for surgical pathologists a new tool capable of correlating the clinical stages of individual cases and their degree of malignancy, including a detailed study of the marginal boundaries of each individual tumor. This task can be achieved with histologic sections of the tumor and its metastases and also by means of quantitative immunochemical techniques. Even if many details of the biological function of p27BBP are not yet fully known, its easily observed or measured overexpression may provide a further instrument in the diagnostic toolkit of surgical pathologists. Moreover, in some tumors, we have identified an additional higher-size protein that is recognized by one antibody to p27BBP that is also found in lymph node metastases. Although the nature of this novel protein is not yet known, we suggest that it may represent a potentially sensitive marker of metastatic foci.

**MATERIALS AND METHODS**

**Patients.** Surgical specimens were obtained from 41 patients (33 men and eight women; mean age, 61 years) who were diagnosed with and underwent resection for squamous cell carcinomas of different sites of the head and neck at the II Otolaryngological Clinic, University of Torino. Written consent for involvement in this study was obtained from each patient before surgical intervention. The tumor stages were T1–T4, N0–N2c, and M0. Upon surgery, hematoxylin and eosin sections were reviewed by two pathologists to confirm diagnosis and to classify tumors as well-differentiated, moderately differentiated, or poorly differentiated squamous cell carcinomas. Any patients who previously underwent surgical treatment for the same carcinoma or other tumors, those who were previously treated with chemotherapy or radiotherapy, and those who had other neoplasias were excluded from this study. The tumor topography and the clinicopathologic characteristics of selected patients are summarized in Table 1.

**Sampling of Normal and Pathologic Tissues.** Normal and pathologic tissues were obtained immediately after surgery from each resected tumor

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<th>Table 1. Patient clinicopathological characteristics.</th>
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Abbreviation: ND, not determined.
specimen. Normal lymph nodes and lymph node metastases were also evaluated in seven patients. For immunohistochemical analysis, small specimen blocks were immediately embedded in OCT 4583 (Miles Scientific, Naperville, IL) and frozen in liquid nitrogen–cooled methylbutane (BDH Laboratory Supplies, England). For molecular and biochemical analysis, sample blocks were immediately frozen in liquid nitrogen. Samples of each surgical specimen were subjected to standard diagnostic procedures according to TNM classification criteria of the International Union Against Cancer.11

**Antibodies.** The rabbit polyclonal antiserum against a C-terminal peptide of p27$^{\text{BBP}}$ (s13) has been previously described2,4 and routinely used for immunochromistry. Routinely, the antiserum was pre-adsorbed with the peptide and used to obtain negative controls. The secondary antibodies goat anti-rabbit biotin-conjugated IgGs were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Rabbit anti-α, γ smooth, γ cytoplasmic actins (AAL20) antisera were generous gifts from G. Gabbiani (University of Geneva, Switzerland).

**Immunohistochemical and Histochemical Staining.** Five-micrometer cryosections were collected on polylysine-coated slides and fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Sections were treated with 0.5% Triton X-100 for 10 min, followed by 0.3% hydrogen peroxide in PBS for 30 min, blocked with normal goat serum for 70 min, and then incubated for 2 h at room temperature with 1:250 anti-p27$^{\text{BBP}}$ polyclonal antibody. After PBS washings and incubation for 80 min at room temperature with the biotin-conjugated secondary anti-rabbit at 1:200, the sections were labeled using the avidin-biotin amplification method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and revealed by horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech) at 1:2000 dilution. Specific bands were revealed by enhanced chemiluminescence (ECL Western blot analysis system; Amersham Pharmacia Biotech). Filters were stripped in 0.3 M NaOH for 5 min and reblotted with the rabbit anti-actins (AAL20) antiserum at 1:1000 dilution and then probed with horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech) at 1:2000 dilution. Specific bands were revealed by enhanced chemiluminescence (ECL Western blotting analysis system; Amersham Pharmacia Biotech).

**Isolation of RNA and Northern Blot Analysis.** Total cellular RNAs were isolated from frozen human tissues, homogenized in liquid nitrogen, and extracted by the acid guanidium thiocyanate–phenol–chloroform method.12 Total RNA was reconstituted in RNAase-free water (Clontech, Palo Alto, CA) and quantified by optical density measurement. Fifteen micrograms of denatured total RNA was electrophoresed on a denaturing formamide-agarose gel and transferred to Hybond-N filter (Amersham Pharmacia Biotech). Hybridization was carried in 50% formamide at 42°C, with 1.5 × 10$^{-6}$ cpm (per mL of hybridization solution) of homologous probe. The probe, corresponding to the open reading frame of p27$^{\text{BBP}}$ (748 bp), was randomly prime labeled with [α-32P]dCTP. After hybridization, the filter was washed twice at 42°C
in 2 × sodium chloride/cytate (SCC)/0.1% SDS, for 20 min. For mild-stringency washes, the filter was then treated once at 42°C in 1 × SSC/0.1% SDS for 20 min. The filter was exposed to −80°C on Hyperfilm (Amersham Pharmacia Biotech) for 24 h. Normalization was performed using a GAPDH cDNA probe.

**Southern Blot Analysis.** Seven micrograms of high-molecular-weight DNA derived from tissues of 10 patients was digested with *Pst*I and *Hind*III (New England BioLabs, Beverly, MA), separated by electrophoresis on 0.8% agarose gel, and transferred to nylon membranes Hybond N⁺ (Amersham-Pharmacia Biotech). The filter was hybridized with 700,000 cpm/mL of [α-32P]dCTP randomly prime labeled full-length p27<sub>BBP</sub> cDNA probe, including the 3' untranslated region of the protein (1080 bp) (Prime-a-Gene Labeling System; Promega, Madison, WI) at 65°C for 16 h in 0.25 M sodium phosphate buffer, pH 6.8, containing 7% SDS and 200 µg/mL denatured sonicated salmon sperm DNA. After hybridization, the membrane was washed once at room temperature in 2 × sodium chloride, sodium phosphate, EDTA (SSPE)/0.1% SDS and then, for high stringency, washed sequentially with 1 × SSPE/0.1% SDS, 0.5 × SSPE/0.1% SDS, 0.1 × SSPE/0.1% SDS for 20 min at 65°C.

**RESULTS**

**Distribution of p27<sub>BBP</sub> in Normal Squamous Epithelia of the Upper Aerodigestive Tract.** Immunohistochemical analysis was performed on 37 samples of normal squamous epithelia using a rabbit antiserum raised against a synthetic peptide of the C-terminus of p27<sub>BBP</sub>.<sup>2</sup> In squamous epithelia, p27<sub>BBP</sub> staining was very marked in the basal layer where the protein was concentrated in a single, dark, and regularly shaped nucleolar dot [Figure 1C–1E (arrow)]. Moving toward the superbasal layers, more than one dot was usually seen in the nucleus, and these showed a tendency to fuse in a single mass [Figure 1C–F (arrowhead)]. p27<sub>BBP</sub> was not detectable in the superficial layers (Figure 1A, 1B, and 1F). As previously shown,<sup>1</sup> the weak specific cytoplasmic staining was abolished by incubating the sections with the anti p27<sub>BBP</sub> antiserum pre-adsorbed with the immunogen. The content of p27<sub>BBP</sub> in underlying stromal cells was much lower and concentrated in a single small, barely visible nucleolar dot without any obvious cytoplasmic staining (Figure 1A and 1B).

**Distribution of p27<sub>BBP</sub> in Squamous Cell Carcinomas of the Head and Neck.** The distribution of p27<sub>BBP</sub> was investigated in 41 head and neck squamous cell carcinomas. The intensity of p27<sub>BBP</sub> staining was highly increased in squamous cell carcinomas and was apparently related to the degree of differentiation (Figure 2). Staining was mostly localized in the nucleolus in the form of large, numerous, and/or irregularly shaped dots. Such dots were generally much larger than the dots of the corresponding normal epithelia and mostly appeared to nearly fill the nucleus. In some cells, such large accumulation of p27<sub>BBP</sub> was extremely aberrant, with extended nuclear defects and marked cell atypias being observed [Figure 2J–2L (arrow)]. In dividing cells, p27<sub>BBP</sub> was obviously associated with individual tiny dots corresponding to mitotic chromosomes [Figure 2M–2O (arrowhead)]. They appeared to be associated to kinetochores at metaphase, and, at anaphase, the dots behaved like chromosomal passenger proteins.<sup>4</sup> In some tumors, displaying a more differentiated phenotype, horny pearls could be identified, mostly with a peripheral distribution of all the protein (Figure 2C). Horny pearls displayed much more p27<sub>BBP</sub>-positive nucleoli in outer cells than in inner cells.

p27<sub>BBP</sub> immunoreactivity in the stromal tissues surrounding tumor nests was low and similar to that observed in normal epithelia, ie, it was virtually absent from the cells observed around the tumor mass (Figure 2A).

Data indicate that p27<sub>BBP</sub> distribution was altered in quantity and located in widely polymorphous masses in carcinoma cells. Such a large increase in the p27<sub>BBP</sub> dot number and size as well as the staining intensity pattern made identification of transformed versus normal cells very easy and unambiguous, even for occasional micrometastatic foci.

According to the semiquantitative evaluation of immunohistochemistry previously described in the Materials and Methods, statistical analysis was performed to compare scores obtained by three independent observers on sections of tumors versus normal mucosa. Fisher’s exact test showed a significant difference between the two groups (χ² = 36.667; p < .0001).

**Distribution of p27<sub>BBP</sub> in Normal and Metastatic Lymph Nodes.** The distribution of p27<sub>BBP</sub> was studied in parallel in seven normal and metastatic lymph nodes. In normal lymph nodes, p27<sub>BBP</sub> was...
found in lymphocytes and plasma cells as a very small, regular, and weakly stained nucleolar dot (Figure 3A), whereas the p27BBP content in high endothelial cells was usually extremely low and only occasionally structured in tiny dots [Figure 3B (arrowhead)].

Against this weakly stained background belonging to lymph node–resident cells, metastatic squamous tumoral cells mixed with lymphoid tissue were promptly and easily recognized on the basis of their higher p27BBP staining signal and their highly prominent nucleoli. Nucleoli in metastatic tumor cells were large irregular dots contributing to cell atypias and giving a polymorphous pattern that was even more marked than that of the corresponding primary tumor within the same individual (Figure 3C–3F). It is then clear that immunohistochemical staining for
**FIGURE 2.** Immunolocalization of p27\textsuperscript{BBP} in head and neck squamous cell carcinomas. (A–I) p27\textsuperscript{BBP} staining in squamous cell carcinomas at progressively lower differentiation degree. Transformed cells exhibit nuclear accumulation of p27\textsuperscript{BBP} when compared with their normal counterparts: p27\textsuperscript{BBP} is altered in transformed cells of carcinomas at different stages and is localized in the nucleus as three or four nucleolar dots with an irregular shape. (C) Horny pearl; as the cells progressively make the horny pearl, expression of p27\textsuperscript{BBP} disappears or decreases. (J–L) Aberrantly large accumulation of p27\textsuperscript{BBP} reflects polyploidy or other cell atypias (arrow). (M–O) p27\textsuperscript{BBP} redistributes with chromosomes at mitosis (arrowhead). Bars denote 40 μm (A, B) and 25 μm (C–O).

\textbf{p27\textsuperscript{BBP} is a rapid and reliable approach to identify tumor cells in lymph nodes that have been colonized by the primary tumor.}

\textbf{p27\textsuperscript{BBP Expression in Carcinomas and Lymph Node Metastases.} On the basis of immunohistochemical data, p27\textsuperscript{BBP} levels were analyzed in 41 carci-}
noma samples by Western blot and compared with corresponding normal mucosa in 37 cases. This paired analysis of blotted samples showed an upregulation of the protein in tumor tissues as earlier observed in other tumor types. In 22 carcinomas (59%), densitometric analysis of paired blotted samples showed an increased expression of p27BBP in tumor versus normal tissues. Within this group, 13 cases (35%) did not show any up-regulation of the protein and two cases (5%) showed a decrease in p27BBP expression (in four cases, normal tissues were not available to compare data). In fact, Western blot data matched immunohistochemistry and showed p27BBP expression to be related with malignancy. Moreover, p27BBP was always significantly overexpressed in metastasis-bearing versus normal lymph nodes.

Strikingly, filter incubation with the p27BBP polyclonal antiserum showed the presence of an additional band located approximately at 52 kDa.
that was never observed in earlier studies.1 This band was not visible in most normal mucosal samples or barely evident in others but was strongly evident in almost all the tumors (40 of 41). More intriguingly, Western blot analysis of metastasis-containing tissues always showed the 52-kDa band that was always absent in the normal lymph nodes from the same patient (Figure 4A and 4C). The 52-kDa band could be abolished by incubating the polyclonal antiserum (s13) with the p27BBP peptide used for the immunization (Figure 4B), indicating that the relevant epitope is within a domain comprising or structurally influenced by the C-terminal sequence of p27BBP. The band was observed in both reducing and nonreducing conditions, indicating that it was not a dimerization artifact due to disulfide bridges (not shown).

Blast analysis failed to find any obvious homologue of p27BBP. To verify whether the 52-kDa band was due to gross genetic alterations of the p27BBP gene or to abnormal alternative splicing of the p27BBP mRNA, Southern and Northern blot analyses were performed on 10 paired normal and tumor tissue samples and on two paired normal and metastatic lymph node samples. All the tumors showed identical band patterns in Southern blot when compared with the corresponding normal mucosa (Figure 5), thus suggesting subtle alterations or the presence of a cross-reactive gene product. Northern blot analysis showed a significant upregulation of p27BBP mRNA in all 10 cases of carcinoma and their metastases, while no evidence of alternative splicing was
found (Figure 6). Taken together, these data suggest that the 52-kDa band is the result of cross-reactivity with unknown genes or is due to abnormal yet unidentified post-translational modifications of p27BBP.

DISCUSSION

The major result of this work is that p27BBP is overexpressed in head and neck carcinomas at a significantly higher extent than in normal mucosa, and this allows an accurate delimitation of the tumor mass, even in comparison with other histochemical procedures. Moreover, tumor cells in metastatic foci can be promptly identified within lymph nodes because prominent nucleolar masses are highlighted by their content of p27BBP. This allows a quick and simplified survey of lymph nodes in postoperative surgical pathology and may also offer a quick intraoperative assay when histochemical detection is performed by a quick fluorescence test. There is no superposition of this study with those reported in our previous paper on colorectal cancers for two reasons: first, the latter tumors were derived from gut columnar epithelium, and we studied head and neck squamous cell carcinomas with the intention to uncover possible differences linked to the nature of the original epithelial type; second, local lymph node effusions in head and neck tumors are much more readily available.

A second result is the unexpected identification of a p27BBP cross-reactive 52-kDa band that has not been previously observed in other regions and their tumors. In particular, we did not find it in a previous study of colorectal carcinomas. Intriguingly, this band is highly overexpressed in carcinomas. Moreover, Western blot analysis was performed on specimens from a group of five adenocarcinomas of the parotid gland and one leiomyosarcoma of the larynx (these patients were not introduced in the group of study). In all of these cases, the 52-kDa band was missing, probably indicating tissue specificity because it has been found so far only in squamous epithelia lining the upper aerodigestive tract. This band cross-reacts with polyclonal antibodies raised to p27BBP synthetic peptides derived from the C-terminus and may be specifically abolished using absorbed antibodies. Thus, the most likely interpretation of its molecular structure is that it is either due to cross-reactivity of the antiserum toward a related yet unidentified gene or to post-translational modifications of p27BBP. Protein purification associated with mass-spectral analysis is needed to solve this problem, provided that sufficient amount of tissue is obtained.

The 52-kDa band is totally absent from lymph nodes (but not from those lymph nodes that contain metastatic foci), and this may indicate (1) a specific expression of this protein species in the airway mucosa (but other districts of the airway mucosa do not express it) or (2) the existence of a few scattered tumor cells or cells undergoing transformation within the normal mucosa close to some tumors that are not otherwise identifiable and may be responsible for local tumor relapse. The local normal mucosa may not be normal at all on the basis of the expressed 52-kDa form, and this opens the possibility that the cell disorder in head and neck tumors may be more extended than previously expected.

The real biological role of p27BBP is not completely clear and is now being actively investigated in this and other laboratories. After its discovery in a search for cytoplasmic interactors of the integrin subunit β4, p27BBP was found to have much broader significance as an evolutionary conserved controller of ribosome assembly and protein synthesis. The significance of the cytoskeleton association of p27BBP is still unexplained. In colorectal cancer, p27BBP has been found to be overexpressed as a function of malignancy, similar to the data reported in the present paper. Overexpression of p27BBP may be a general feature of most or all malignant tumors (manuscript in preparation), and the molecular determinants of its overexpression may be searched in the structure of its gene promoter or

![FIGURE 6. p27BBP expression analyzed by Northern blotting. In normal (N) and neoplastic (Ca) tissues and in normal (NLn) and metastatic (MLn) lymph node tissues, a 1100-bp transcript of p27BBP is detected that is highly increased in tumors and metastases compared with their normal counterparts.](image-url)
in the location of the gene at 20q11.2, a highly unstable region of the human genome. In conclusion, we report that p27BBP is overexpressed in head and neck cancers and its metastases but not in the corresponding normal mucosa sampled from the same individual patients. This overexpression is statistically highly significant. Moreover, we report the observation of a related 52-kDa form, apparently specific for malignant tissues. Future studies will aim to identify the 52-kDa band and to determine the usefulness of this reagent as a diagnostic tool used by head and neck surgeons and surgical pathologists also to delimit the margins of individual tumors.

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REFERENCES