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Advances in Brief

Overexpression of CDC25A and CDC25B in Head and Neck Cancers[1]

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Abstract

The deregulation of several cell cycle-related genes participates in neoplastic transformation. Cell cycle progression is driven by cyclin-dependent kinases, which are positively regulated by association with cyclins and negatively regulated by binding to inhibitory subunits. The activity of cyclin-dependent kinases is also regulated by the phosphorylation status, which is controlled by the antagonistic action of wee1 kinase and CDC25 phosphatases. Three *CDC25* genes are present in human cells: *CDC25A*, *CDC25B*, and *CDC25C*. These three genes function at different phases of the cell cycle. Whereas CDC25A and CDC25B are expressed throughout the cell cycle, with peak expression in G_1 for CDC25A and in both G_1-S-phase and G_2 for CDC25B, CDC25C is predominantly expressed in G_2. Several lines of evidence suggest a role for CDC25s as oncogenes. CDC25A and CDC25B cooperate with Ha-*ras* or loss of *Rb1* in the oncogenic transformation of rodent fibroblasts. Moreover, they are transcriptional targets of c-*myc*, and CDC25A in particular plays an important role as a mediator of *myc* functions. On the basis of the evidence that CDC25 phosphatases can act as oncogenes, we analyzed the expression of *CDC25A*, *CDC25B*, and *CDC25C* genes in 20 squamous cell carcinomas of the head and neck by quantitative reverse transcription-PCR. Our results show that whereas CDC25C is expressed at a low level with no relevant differences between neoplastic tissue and normal mucosa, CDC25A and CDC25B are overexpressed in a large fraction of tumors.

Introduction

Head and neck cancers are the sixth most common neoplasm in the world (1). They include cancers of different sites, such as the larynx, pharynx, oral cavity, and tongue, and over 90% of them are HNSCCs.[3] Epidemiological surveys have shown tobacco smoking as a major risk factor in the development of HNSCCs, but other factors, such as alcohol consumption and environmental exposure, play a synergistic role (2, 3). It is known that cancer arises as a consequence of a series of genetic changes that are followed by clonal expansion of cells through a selective growth advantage. The identification of the nature and timing of these changes is critical for the biological understanding of the disease and can provide markers useful for diagnosis and prognosis.

Accumulating evidence suggests that the deregulation of several cell cycle-related genes participates in neoplastic transformation. In eukaryotes, cell cycle progression is controlled by a family of enzymes known as CDKs (4). These enzymes consist of a catalytic subunit (CDK) that is positively regulated by association with a regulatory subunit, known as cyclin, and negatively regulated by binding to inhibitory subunits (p16, p21, p27, and others). The activity of CDKs is also regulated by phosphorylation; inhibitory phosphorylation of threonine and tyrosine residues in the ATP-binding site inhibits kinase activity. These phosphorylation sites are conserved among eukaryotes, and their phosphorylation status is controlled by the antagonistic action of wee1 kinase and CDC25 phosphatases (4).

In human cells, three *CDC25* genes, *CDC25A*, *CDC25B*, and *CDC25C*, have been isolated (5–7). These three *CDC25* genes function at different phases of the cell cycle. *CDC25A* and *CDC25B* are expressed throughout the cell cycle with a peak expression in G_1 3–5 h after serum stimulation for *CDC25A* and in both G_1-S-phase and G_2 for *CDC25B* (6, 8). *CDC25C* is predominantly expressed in G_2 and regulates the timing of entry into mitosis (7, 9).

Several lines of evidence suggest a role for CDC25s as oncogenes. CDC25A and CDC25B have been shown to cooperate with either mutated versions of Ha-*ras* or loss of *Rb1* in oncogenic transformation of murine fibroblasts. The transfected cells can form colonies in soft agar and induce the formation of tumors when injected in nude mice (10). The *CDC25A* and *CDC25B* genes are transcriptional targets of the c-*myc* oncogene, and CDC25A in particular plays an important role as a mediator of *myc*-induced cell cycle activation as well as apoptosis (11). Moreover, expression levels and phosphatase activity of CDC25A are strongly induced in fibroblasts by the adenovirus E1A oncoprotein, indicating that a high level of CDC25A presumably contributes to the ability of E1A to override the checkpoint control mechanisms during the G_1 phase to induce DNA replication (12). Finally, CDC25A and CDC25B are overexpressed in some cancer cell lines (6) and in 32% of human breast cancers,[4] suggesting a role for these genes in neoplastic transformation.

In this study, the role of *CDC25* genes in head and neck cancer development was analyzed by using a highly sensitive quantitative RT-PCR approach. Moderate or low levels of expression were detected for the *CDC25C* gene, with no relevant differences between neoplastic tissue and normal mucosa. In contrast, *CDC25A* and *CDC25B* were significantly overexpressed in a large fraction of tumors, suggesting that a deregulated expression of these CDC25 phosphatases may play an important role in head and neck cancer development.

Materials and Methods

Samples. Twenty squamous cell carcinomas and corresponding normal mucosa of the upper aerodigestive tract were collected from patients at the City Hospital of Pordenone. No patient had been treated with chemotherapy or radiotherapy before surgery. All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80°C until extraction of RNA.

Quantitative PCR Analysis. Total cellular RNA was extracted using the guanidinium thiocyanate method (13). Evaluation of gene expression by Northern blot analysis in fresh tumors is often prevented by the fact that insufficient amounts of RNA are recovered from these samples. In addition, accurate analysis of genes expressed at a low level or of large transcripts, like CDC25 mRNAs, requires very high-quality RNAs, because even a small amount of degradation may impair the quantification of the transcript. To circumvent these limitations, we adopted a very sensitive and quantitative

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[3] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; CDK, cyclin-dependent kinase; RT-PCR, reverse transcription-PCR.

[4] K. Galaktionov, personal communication.

PCR-based analysis, competitive RT-PCR (14). In a competitive RT-PCR, a constant amount of reverse-transcribed RNA is mixed and coamplified with fixed amounts of a synthetic competitor that differs from the target cDNA for the presence of a small insertion/deletion or a restriction site, such that the two products are easily distinguishable by gel electrophoresis. Because of the high similarity, the two products are amplified with the same efficiency, and the amount of target cDNA is quantified on the basis of the amount of synthetic competitor needed to produce an equivalent PCR signal. Differences in the amount of starting RNA as well as in reverse transcriptase efficiency in diverse samples are minimized by normalizing data in comparison with the expression of a housekeeping gene (in the present study, the ribosomal protein S14 gene), also evaluated by competitive PCR. The system was standardized comparing PCR and Northern blot data obtained from a series of head and neck-derived cell lines that showed different expression levels of CDC25 mRNAs (data not shown).

For RT-PCR, 1 μg of RNA was reverse-transcribed in a 20-μl reaction by random hexanucleotide priming using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions.

RNA expression analysis was carried out by a competitive PCR method using synthetic competitors that differed from the cDNA of interest by having a small internal insertion. The primers used, the annealing temperature, and the size of amplified fragments are reported in Table 1. The synthetic competitors were generated by overlap extension (15). The CDC25A synthetic competitor contained a 14-bp insertion between nucleotides 1425 and 1426 [the numeration is according to Galaktionov *et al.* (5)]. The competitor for CDC25B carried a 20-bp insertion between nucleotides 1266 and 1267 [the numeration is according to Galaktionov *et al.* (5)]. The competitor for CDC25C contained an insertion of 18 nucleotides between nucleotides 650 and 651 [the numeration is according to Sadhu *et al.* (7)]. The competitor for RPS14 contained an insertion of 16 nucleotides between nucleotides 3021 and 3022 [the numeration is according to Rhoads *et al.* (16)].

PCRs were performed in a total volume of 20 μl using a constant amount of cDNA (1 μl of the cDNA previously diluted 5-fold) that was coamplified with standard dilution series of the competitor, 10 pmol of each primer, buffer, and Taq DNA polymerase (Promega), according to the manufacturer's instructions. The PCR reactions were carried out for 43 cycles (CDC25A, CDC25B, and CDC25C) or for 30 cycles (RPS14). The amplified products were separated by electrophoresis in a 4% agarose Tris-borate EDTA gel, and the bands were visualized by ethidium bromide staining and quantified by densitometric scanning. The concentration of the target cDNA was extrapolated from a log/log plot of the ratio of competitor:cDNA *versus* the known concentrations of input competitor. Differences in the amount of starting RNA as well as in reverse transcriptase efficiency in diverse RT reactions were minimized by normalizing data in comparison to the expression of the housekeeping gene ribosomal protein S14, also evaluated by competitive PCR. Cases were considered overexpressing when the ratio between expression in the tumor and expression in normal tissue was ≥ 3.

Results and Discussion

The expression of *CDC25* genes was analyzed in 20 head and neck tumors and corresponding normal mucosa by a highly sensitive quantitative PCR. In normal mucosa, the three genes were expressed at a low level [mean values and SD: CDC25A, $(4.5 \pm 0.9) \times 10^3$ copies/μg of RNA; CDC25B, $(4.0 \pm 1.0) \times 10^4$ copies/μg of RNA; and CDC25C, $(5.0 \pm 1.2) \times 10^3$ copies/μg of RNA], and no relevant difference in the expression level of CDC25A, CDC25B, or CDC25C was detected among the different samples. Sixteen of 20 tumors analyzed (80%) exhibited increased expression of CDC25A mRNA when compared with the adjacent normal tissue. In these tumors, the level of CDC25A was 3–12-fold the expression of corresponding normal mucosa (Table 2 and Fig. 1), with 55% of the cases showing overexpression levels above 5-fold. Overexpression of CDC25B mRNA was detected in 50% of the cases, with the expression levels in tumors 3–5.5-fold higher than those in normal mucosa. In contrast, no significant difference between tumors and normal mucosa was observed for the *CDC25C* gene (Table 2). Our results provide the first evidence of CDC25A and CDC25B overexpression in human HNSCCs.

Table 2 *CDC25 mRNA expression*

Case	CDC25A expression (tumor/normal mucosa)	CDC25B expression (tumor/normal mucosa)	CDC25C expression (tumor/normal mucosa)
HN2	1.5	1.2	1.2
HN4	4.5	1.2	1
HN5	2.5	2.2	0.8
HN7	5.5	2.5	1
HN10	3.5	3.8	1.5
HN16	5.1	3.1	1.5
HN18	10	2.5	2
HN20	5.4	3	1.5
HN28	3	1.5	1
HN30	10	5	1.2
HN32	8	3.8	1.5
HN58	12	2.5	0.7
HN139	3.6	3.4	1
HN158	4.5	5.5	1.5
HN182	5.2	2	0.8
HN189	10	2	1
HN191	7	4	1.2
TC50	1.2	1.4	1
TC51	5.1	3.5	1.5
TC53	1.5	4	1.2
Total	1%20 overexpressing[a]	1%20 overexpressing[a]	

[a] Cases were considered overexpressing when the ratio between expression in the tumor and expression in normal tissue was ≥ 3.

The expression of CDC25B and CDC25C has been previously analyzed by Nagata *et al.* (6) in some cancer cell lines by Northern blot. These investigators reported that CDC25B was particularly overexpressed in the T24 bladder carcinoma cell line and in a cell line of SV40-transformed fibroblasts, whereas CDC25C was expressed at a low level without any obvious difference in the level of expression among the different cell lines. A high level of CDC25A and CDC25B expression in cell lines was also reported by Galaktionov *et al.* (10); in their paper, the expression of CDC25B and CDC25C was also investigated in a series of human breast cancers by *in situ* hybridization, and whereas no CDC25C mRNA was detected in normal or tumor tissues, CDC25B was overexpressed in 32% of the neoplastic samples (10).

Table 1 *PCR primers*

Gene	Primer sequence[a]	Annealing temperature (°C)	Amplified fragment (size)	Location (reference)[b]
CDC25A	*sense*, GAGGAGTCTCCACCTGGAAGTACA *anti*, GCCATTCAAAACAGATGCCATAA	56	272	nt 1297-1569 cDNA (5)
CDC25B	*sense*, CACGCCCGTGCAGAATAAGC *anti*, ATGACTCTCTTGTCCAGGCTACAGG	59	416	nt 1059-1475 cDNA (5)
CDC25C	*sense*, CAGGAAGTGCATTTAGCTGGGATG *anti*, ATCGACGGGGAGCGATATAGGC	60	337	nt 523-860 cDNA (7)
RPS14 (ribosomal protein S14)	*sense*, TGTGACTGGTGGGATGAAGGT *anti*, CAGGTCCAGGGTCTTGGTCC	61	167	nt 2917-4186 DNA (16)

[a] anti, antisense.
[b] nt, nucleotides.

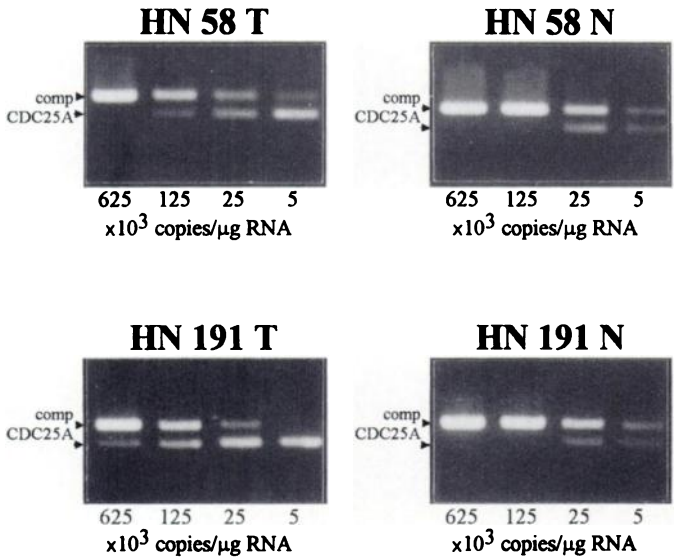

Fig. 1. Competitive PCR analysis of CDC25A mRNA expression in HNSCCs. For each sample, constant amounts of reverse-transcribed RNA were amplified together with a dilution series of the CDC25A synthetic competitor. The amplified products were separated by agarose gel electrophoresis (competitor, *upper band*; cDNA, *lower band*), and the ratio of competitor:cDNA was quantified by densitometric analysis. Differences in the amount of starting RNA as well as in reverse transcriptase efficiency in diverse samples were minimized by normalizing data in comparison with the expression of the ribosomal protein S14 gene, which was also evaluated by competitive PCR.

In light of these findings and of the *in vitro* evidence that CDC25A and CDC25B can act as oncogenes, our data suggest a role for CDC25 overexpression in head and neck cancer development.

Increasing evidence suggests a key role for cell cycle-related molecules in the transformation of cells of the upper aerodigestive tract; cyclin D1 is overexpressed in about 50% of the carcinomas of the upper aerodigestive tract (17), and p53, a well-known G_1 checkpoint molecule, is mutated in about 60% of the cases (18). Moreover, p16 inactivation has been detected in over 80% of these tumors (19). Our data add another piece to the puzzle of events contributing to the neoplastic transformation of cells of the respiratory tract and underline, once again, the important role of molecules involved in the control of G_1-S-phase transition.

The mechanisms by which overexpression of CDC25s would contribute to neoplastic transformation seem to be diverse, although they are not fully understood. It is known that CDC25 phosphatases play a role in cell cycle progression by the activation of CDKs. The physiological substrates of each member of the CDC25 family have yet to be precisely determined; however, CDC25C has been implicated in the dephosphorylation of the mitotic kinase cdc2 (CDK1), and recent evidence suggests the G_1-S-phase kinase CDK2 and possibly CDK4 as the most likely candidate substrates of CDC25A and CDC25B (11, 20).

On the other hand, CDC25A and, to some extent, CDC25B were found to associate with raf-1 at or near the plasma membrane and to be phosphorylated by raf-1, an event associated with the activation of CDC25 enzymatic activity, indicating that CDC25 phosphatases are involved in linking cell cycle control to the mitogenic signal transduction pathway (21, 22).

The recent finding that c-*myc*, a transcription factor involved in the control of normal cell proliferation and in the induction of neoplasia, is a direct transcriptional activator of CDC25A and CDC25B suggests a role for these genes as mediators of *myc* function (11). Interestingly, about 30–70% of head and neck cancers overexpress the c-*myc* gene (23), suggesting that overexpression of CDC25A and CDC25B in these tumors may be a consequence of c-*myc* deregulation, and therefore mediate c-*myc* oncogenic properties.

Finally, despite the different protocols that have been tested in the therapy of head and neck tumors, aggressive surgery and radiotherapy still constitute the treatments of choice for this tumor type, which shows a certain degree of unresponsiveness to chemotherapy. Recent findings show that the mechanisms of action of some benzoquinoid antitumor compounds may involve CDC25B inactivation (24). The identification of the tumor types in which this gene is overexpressed could help to identify the neoplasms that are likely to be more sensitive to these chemotherapeutic treatments.

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