Overexpression of CDC25A and CDC25B in Head and Neck Cancers

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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 G1 for CDC25A and in both G1-S-phase and G2 for CDC25B, CDC25C is predominantly expressed in G2. Several lines of evidence suggest a role for CDC25A as oncogenes. CDC25A and CDC25B cooperate with Ha-ras or loss of Rbl 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. 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 G1, 3–5 h after serum stimulation for CDC25A and in both G1-S-phase and G2 for CDC25B (6, 8). CDC25C is predominantly expressed in G2 and regulates the timing of entry into mitosis (7, 9).

Several lines of evidence suggest a role for CDC25A as oncogenes. CDC25A and CDC25B have been shown to cooperate with either mutated versions of Ha-ras or loss of Rbl 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 G1 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, 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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1 To whom requests for reprints should be addressed.

2 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.

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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 4 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 ± 0.9) × 10^2 copies/µg of RNA; CDC25B, (4.0 ± 1.0) × 10^4 copies/µg of RNA; and CDC25C, (5.0 ± 1.2) × 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.

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 over-expressed 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>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequencea</th>
<th>Annealing temperature (°C)</th>
<th>Amplified fragment (size)</th>
<th>Location (reference)b</th>
</tr>
</thead>
</table>
| CDC25A          | sense, GAGGAGTCTTCCACCTGGAAAGTACA  
|                 | anti, GCCATTTCAAAACGAGTCCATAA   | 56                        | 272                   | nt 1297-1569 cDNA    |
| CDC25B          | sense, CACCGGGCTGGAGAAAGAGGC  
|                 | anti, ATGCACTCTCGTGCAGACTACAGG  | 59                        | 416                   | nt 1059-1475 cDNA    |
| CDC25C          | sense, CAGGAAGTCTTCTCTTGCTGAAAGTGC  
|                 | anti, ATCGACGGGAGGCGCATATGGCG  | 60                        | 337                   | nt 523-86 cDNA       |
| RPS14 (ribosomal protein S14) | sense, TGTGACTGTTGGGGGAAAGGT  
|                 | anti, CAGTTTCCAGGCGTTTGGGC  | 61                        | 167                   | nt 2917-4186 DNA     |

* anti, antisense.

b nt, nucleotides.
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.

### References


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