Oesophageal cancer and amplification of the human cyclin D gene

CCND1/PRADI

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Summary The human CCND1/PRADI gene, located in the 11q13 chromosomal region, encodes a cyclin D protein with potential oncogenic capacity and is involved in several human malignancies. The amplification and expression status of CCND1 was investigated in a series of oesophageal tumours. CCND1 is amplified in 54% and overexpressed in 63% of the tumours of the squamous cell type.

Keywords: oesophageal cancer; cyclin; chromosome 11; amplification; oncogene

Oesophageal cancer is a frequent and deadly disease linked to environmental factors and resulting from multiple genetic abnormalities primarily identified in the malignant cells. Alterations of oncogenes as well as of tumour-suppressor genes (Evans, 1993) have been observed in oesophageal tumours (Huang et al., 1992; Jankowski et al., 1992). A frequent mechanism of alteration is the amplification of an oncogene locus, resulting in the existence of a high number of copies of a key gene and the overproduction of its messenger RNA and protein product (for review see Brison, 1993). In oesophageal cancer, amplification of several oncogenes has been reported (for review see Yoshida et al., 1993). Thus, MYC, ERBB1, encoding the epidermal growth factor (EGF) receptor and potential oncogenes from the 11q13 chromosomal region can be found amplified in a high proportion of cases (Hollstein et al., 1988; Lu et al., 1988; Tsuda et al., 1989; Kitagawa et al., 1991; Wagata et al., 1991; Jiang et al., 1992; Mori et al., 1992).

The 11q13 chromosomal region, which is amplified in a number of carcinomas (for reviews see Lammie and Peters, 1991; Gaudray et al., 1992; Brison, 1993), rearranged in B-cell (Withers et al., 1991; Williams et al., 1993) and parathyroid tumours (Motokura and Arnold, 1993) and altered in multiple endocrine neoplasia type 1 (Bale et al., 1991; Janson et al., 1991), contains several growth regulator genes. Among these, the CCND1 gene (also called PRADI or CYCD1, and representing the coding unit of the BCL1 locus) encodes a molecule of the cyclin D family (for reviews see Matsushima et al., 1991; Motokura et al., 1991; Xiong et al., 1991; Motokura and Arnold, 1993), which is thought to play a key role in the amplification (Lammie et al., 1991). However, amplification of the 11q13 region appears to result from, or to generate, complex genomic processes. Thus, in addition to CCND1/PRADI, potential unidentified oncogenes of three other 11q13 subregions, either slightly centromeric or telomeric of the cyclin gene (Szpetowski et al., 1991, 1992; Brooks et al., 1993; Karlseder et al., 1994), are suspected to be selected in some of the amplification units. They are close to the D11S97, EMSI (Schuring et al., 1992) and GARF (Olendorf et al., 1994) loci. Finally, another poorly understood characteristic of the 11q13 amplification, only established so far in breast tumours, is its frequent association with an amplification of the 8p12 chromosomal region (Theillet et al., 1993).

The role of the FGF3 and FGF4 genes, encoding growth factors of the fibroblast growth factor family and localised slightly telomeric of CCND1 (Hagemeijer et al., 1991; Brookes et al., 1993), is no longer considered important for the development of the 11q13 amplification units (discussed in Lammie and Peters, 1991, and Gaudray et al., 1992) but the initial observations of 11q13 amplification in oesophageal carcinomas were done with probes for the FGF genes FGF3/INT2 and FGF4/HST. Based on their amplification, a high incidence of alteration of the 11q13 region was observed in oesophageal cancer (Tsuda et al., 1988; Kitagawa et al., 1991; Wagata et al., 1991). The same incidence was later found with a CCND1/PRADI probe (Jiang et al., 1992). In two cell lines with 11q13 amplification, it was possible to observe that the CCND1 amplification, but not the FGF4 amplification, was associated with a high level of expression (Jiang et al., 1992). No such correlation was possible with primary tumours using Northern blot hybridization but a recent study reports the altered expression of the CCND1 protein in 11q13-amplified oesophageal tumours (Jiang et al., 1993).

We have looked for amplification of CCND1/PRADI in a panel of oesophageal carcinomas. To assess the actual involvement of the cyclin D1 gene in the amplification process, we have compared its expression with its number of copies using Northern and Southern blot hybridisations. Adding strength to the hypothesis viewing CCND1 and a main target of 11q13 amplification, a strong correlation was observed between CCND1 RNA expression and gene amplification.

Materials and methods

Tumour samples

A panel of 55 oesophageal tumours and seven samples of normal oesophagus was collected, prior to treatment, over the past 2 years at the Institut Paoli-Calmettes in Marseille. Endoscopic biopsy specimens were frozen within 15 min of removal and were stored at −80°C before processing. Tumours were classified as squamous cell carcinomas (n = 44) and adenocarcinomas (n = 11). The status of p53 and EGFR proteins were determined by immunohistochemistry (Monges et al., 1994). The mammary carcinoma cell lines MDA-MB.134 and MDA-MB.231, used as controls of chromosomal 11q13 amplification (Laforge et al., 1992), were obtained through the American Type Culture Collection and grown according to its recommendations.

Molecular probes

The CCND1 probe was a 1.1 kb EcoRI cDNA fragment derived from a human placenta library using a synthetic oligonucleotide probe corresponding to the CCND1/PRADI
gene sequence (Raynaud et al., 1993) and was a gift from P. Gaudray (Nice, France). The ETS1 probe, located at 11q23, was used as control for loading in Southern blot experiments. It was a 0.75 kb HindIII fragment from plasmid pHES5.4 (De Taisne et al., 1984). The FGFR4/HST and GARP/D11S833E probes, both located at 11q13 telomeric of CCND1, were a 0.8 kb EcoRI–SacI genomic fragment (Adélaïde et al., 1988) and a 2.4 kb EcoRI genomic fragment (Ollendorff et al., 1992) respectively. The Gapdh probe used in control of RNA hybridisations was derived from a mouse clone (Galland et al., 1990).

DNA and RNA analyses

DNA and RNA extractions were performed as follows. The frozen tumour samples were reduced to powder using a Spex 7000 (Bioblock Scientific) in the presence of guanidinium isothiocyanate. The powder was then heated to 50°C for 15 min. The resulting solution was centrifuged for 3 h at 50 000 r.p.m. The RNA was obtained from the pellet, resuspended in distilled water and stored before use. The DNA present in the supernatant was treated as previously described (Theillet et al., 1989). Southern and Northern blot hybridisations were performed as described by Theillet et al. (1993). The levels of amplification were quantitatively assessed by densitometry scanning (LKB) by comparison with the control probe and normal tissue. Owing to a possible dilution of the tumoral component by stromal tissue (each sample was derived from several pooled biopsies) a cut-off value of 2-fold was chosen for amplification.

Results

Amplification of CCND1 in human oesophageal tumours

The amplification status of the CCND1 gene was assessed by Southern blot analysis in a panel of 35 DNAs extracted from oesophageal tumour samples. The results are shown in Table I and examples of Southern blot hybridisation are shown in Figure 1. Twenty-four squamous cell tumours out of 44 (54%) showed amplification of the CCND1 gene. Compared with normal oesophagus, the number of gene copies was increased between 2 and 12 times. Amplification of at least 3-fold occurred in 45% of the tumours. Amplification was only observed in squamous carcinomas; none of the 11 adenocarcinomas exhibited amplification of CCND1 (Table I). A probe for the ETSI gene, located at 11q23, was used as a control for DNA loading. ETSI was not amplified. Probes for the FGFR4 and GARP genes (Ollendorff et al., 1994), located at 11q13.3 (Adélaïde et al., 1988; Hagemeijer et al., 1991) and 11q13.5–q14 (Ollendorff et al., 1992) respectively, were used to estimate the size and structure of the amplification units (Figure 1). FGFR4 was amplified in 11 cases out of 42 tested (26%). All cases showed amplification of CCND1. GARP was amplified in 10 cases out of 42 (23.7%). In three of these cases, CCND1 was not amplified. Thus, there does not seem to exist fundamental qualitative differences between 11q13 amplifications in oesophageal and breast cancers since co-amplification of FGFR4 with CCND1 and independent amplification of GARP are observed (Karl-seder et al., 1994) but the overall frequency of amplification is higher.

Expression of CCND1 in 11q13-amplified tumours

Whether the amplification of CCND1 contributed to an elevated expression was determined by comparison of RNA expression with DNA amplification. We analysed the expression of the CCND1/GARP gene by Northern blot hybridisation of total RNAs extracted from 38 oesophageal tumours in which both RNA and DNA could be analysed. Examples of hybridisation are shown in Figure 2. The CCND1 gene was expressed as a 4.5 kb transcript. In the vast majority of tumours without CCND1 amplification (16/38), absence or very low levels of expression were observed. Rare tumours, however, showed a significant level of expression in the absence of DNA amplification. In tumours presenting amplification of the gene, CCND1 expression was always observed (24/38). The results are summarised in Tables I and II.

Thus, in the normal oesophagus, the level of expression of CCND1 is either low (and barely detectable by Northern blot analysis) or totally absent. Under pathological conditions, when the gene is amplified, the level of expression becomes readily detectable.

Table I Amplification and overexpression of CCND1 in human oesophageal tumours

| Tumours                | Amplification | Overexpression | A + E |
|------------------------|---------------|----------------|-------|
| Squamous cell carcinomas | 24/44 (54%)   | 24/38 (63%)    | 22+   |
| Adenocarcinomas         | 0/11          | 3/7            | 0     |
| Normal oesophagus       | 0/7           | 0/5            | 0     |
| Normal stomach          | 0/1           | 0/0            | 0     |

*Amplified and overexpressed. **Two non-amplified tumours (including 4523 shown in Figure 2) expressed CCND1 at a significant level, and all amplified tumours overexpressed CCND1 but two amplified tumours could not be tested for expression (see details in Table II).
Correlations with clinical and pathological parameters

Statistical analysis of the correlations between DNA amplification and expression of CCND1 and clinical and pathological parameters was performed on the panel of oesophageal tumours. The results are summarised in Table III. There was no obvious strong statistical correlation between amplification, or expression, and any of the clinical or biological parameters tested. There was no significant association between CCND1 amplification (not shown) or expression (Figure 3) and long-term survival (>24 months).

Discussion

The involvement of cyclin genes in human cancer has recently been shown by several authors. The human cyclin D1 gene, CCND1/PRAD1, is thought to be the key gene in the 11q13 amplification observed in several types of human cancers, as well as the long-sought BCL1 oncogene (Hunter and Pines, 1991; Motokura and Arnold, 1993). This is based on several observations, in particular: (i) the localisation of CCND1 in the major core of the amplified region (Karlseder et al., 1994); (ii) a good correlation between expression and amplification of CCND1 in tumours with an 11q13-amplified region (Lammie et al., 1991); (iii) the consistent expression of CCND1 in lymphomas with a t(11;14) translocation (Rosenberg et al., 1991; Withers et al., 1991); and (iv) the capacity for CCND1 to be activated by tumoral rearrangements (reviewed in Motokura and Arnold, 1993). Furthermore, cyclin D2 and cyclin E genes have been found to be amplified in some human tumours (Buckley et al., 1993; Keyomarsi and Pardee, 1993; Leach et al., 1993), and cyclin A can be activated by provirus insertion in liver cancer (Wang et al., 1990). Finally, even in the absence of amplification or translocation, various cyclin genes are overexpressed in a number of tumours (Buckley et al., 1993; Keyomarsi and Pardee, 1993). The present report strengthens these observations. It illustrates the possible involvement of CCND1 in squamous cell carcinomas of the oesophagus. The incidence of amplification and overexpression of CCND1 in this type of tumour is high, between 45 and 54% depending on the threshold retained for a bona fide amplification. This was expected since it had already been observed that amplification of the chromosomal region where CCND1 is located is especially frequent in this type of cancer (Tsuda et al., 1989; Kitagawa et al., 1991; Wagata et al., 1991). The proportion of oesophageal tumours amplified for probes of the 11q13 region varies from 24% (Mori et al., 1992) to 52% (Tsuda et al., 1989). The lowest incidence, found with an FGF3 probe, is close to what we observed using FGF4 as a probe. 

Mori et al. (1992) observed an association between CCND1 amplification and we were unable to confirm this, although the trend in our data is in a similar direction. At any rate, although almost two-thirds of the squamous cell carcinomas overexpress CCND1, any effect on the survival of the patients is seen only after 12 months. Whether this reflects the initial presence of involved lymph nodes or a enhanced aggression of the carcinoma cells themselves remains unclear at the present time.

Table III Correlation between CCND1 amplification and prognostic parameters

| Criteria               | Number of cases | Number of amplified cases | P-value |
|------------------------|-----------------|----------------------------|---------|
| Tumour size            |                 |                            |         |
| < 3 cm                 | 6               | 5                          | 0.18    |
| > 3 < 4 cm             | 13              | 4                          |         |
| > 4 cm                 | 24              | 15                         |         |
| Nodal status           |                 |                            |         |
| Negative               | 21              | 8                          | 0.03    |
| Positive               | 20              | 15                         |         |
| DNA index              |                 |                            |         |
| Diploid                | 14              | 10                         | 0.22    |
| Aneuploid              | 30              | 14                         |         |
| S-phase (%)            |                 |                            |         |
| < 8                    | 1               | 1                          | 0.60    |
| 8 < 12                 | 4               | 2                          |         |
| > 12                   | 39              | 21                         |         |
| EGFR status            |                 |                            |         |
| Weak                   | 22              | 12                         | 0.88    |
| Medium                 | 5               | 3                          |         |
| Strong                 | 8               | 5                          |         |
| P53 status             |                 |                            |         |
| Negative               | 16              | 9                          | 0.83    |
| Positive               | 17              | 10                         |         |

Table II Comparative status of CCND1 amplification and expression in 38 squamous cell carcinomas of the oesophagus

| RNA expression | Normal copy number | Amplified | Total |
|----------------|--------------------|-----------|-------|
| Absence or low level of expression | 14 | 0 | 14 |
| Expression | 2 | 22 | 24 |
| Total | 16 | 22 | 38 |

Figure 2 Expression of CCND1 in human oesophageal tumours. Ten micrograms of total RNA extracted from oesophageal carcinomas and two breast carcinoma cell lines was analysed by Northern blot hybridisation (see Materials and methods) with a CCND1 probe. A transcript of 4.5 kb was observed in some tumour samples, including the two cell lines used as controls. The Gapdh probe was used as a control. Black squares indicate that the CCND1 gene is amplified in the corresponding tumour. All tumour samples are from squamous cell carcinomas with the exception of the adenocarcinoma sample 4969, shown for comparison.

Figure 3 Outcome of 38 patients with squamous cell carcinoma of the oesophagus. The percentage overall survival is plotted against time (in months). The difference between the survival of patients with no detectable CCND1 expression (- - -) (by Northern blot analysis, see text) and that of patients with CCND1 expression (---) (n = 24) does not reach statistical significance (P = 0.4).
The exact role of CCND1 should be discussed with respect to the general complexity of the amplification process. Amplified units can be large and can contain several categories of genes. In addition to amplified but not over-expressed ‘silent passenger’ genes and ‘unwanted passengers’, which may have a negative effect on cell proliferation or on the co-ordination of the amplification and which are eliminated, at least one pathologically relevant oncogene is assumed to be present in an amplification unit. It corresponds to the selected key ‘driver’ gene. In certain cases more than one gene may be selected to create independent amplicons within the same large region (Szepekowski et al., 1992; Karlseder et al., 1994). CCND1 seems to be a key gene of the 11q13 amplification, and the data reported here, showing a good correlation between amplification and expression, strengthen this hypothesis. However, another category of genes is the ‘opportunist passenger’ genes. They are present in the amplification unit but are not primarily selected and responsible for the amplification. They become deregulated and are overexpressed as a consequence of the elevated gene copy number. CCND1 may also belong to this category, although evidence is mounting that it actually represents a key oncogene (see Motokura and Arnold, for a review).

In the cases with overexpression without increased gene copy number, it is possible that the method of Southern blotting used is not sensitive enough. This question could be solved by using other methods, such as fluorescence in situ hybridisation on chromosomes (Kallioniemi et al., 1992).

Alternatively, in the adenocarcinomas, it may result from a mechanism of regulation which is intrinsically different from the squamous subtype.

Cyclins and other components of the cell cycle, together with regulators of genome integrity and cell survival (Hunter, 1993; Lanfrancone et al., 1994), represent ‘cancer genes’ which may be as relevant to tumour development as the so-called classical oncogenes. The analysis of their involvement in human cancer is of primary importance. Thus, the possible role of other cyclins and cell cycle components in oesophageal tumours should continue to be analysed. The association with possible amplification of other chromosomal regions should also be investigated.

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