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Gene amplifications in advanced-stage human prostate cancer

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Abstract Gene amplification is a model of proto-oncogene alterations occasionally observed in human tumors. This amplification can, in some cases, have prognostic value (N-*myc* in neuroblastoma, c-*erbB2* and *int-2* in breast cancer, etc.). Amplifications of the proto-oncogenes c-*myc*, c-*erbB2* and *int-2* have not yet been report in prostate adenocarcinoma, which, like breast cancer, is hormone dependent. We sought amplifications of these three proto-oncogenes by means of Southern blotting in 15 human prostate adenocarcinoma specimens, most of which were advanced (7 stage C and 6 stage D1 or D2). We confirmed the lack of c-*myc* and c-*erbB2* amplification, regardless of the stage, in contrast to the case of breast cancer. *Int-2* amplification was observed in one advanced tumor with bone metastases, out of a total of six stage D tumors. The precise frequency of *int-2* amplification and its role in prostate carcinogenesis remain to be determined.

Key words Prostate carcinoma · Prostatic neoplasms
Gene amplifications · Oncogenes
Advanced-stage disease

Adenocarcinoma of prostate is the most frequent cancer in older men, but its incidence varies greatly from one country to another: for example, it is 15–20 times less frequent in Japan than in the United States and Scandinavia [6]. Epidemiological studies have failed to identify etiologic factors, but some environmental factors appear to play a role in tumor progression [14], and analysis of familial cases suggests the existence of genetic predisposition [29]. Despite attempts at early detection, prostate cancers is often diagnosed at an advanced stage: about 28% of patients have extraprostatic involvement, and 25% have bone metastases [15]. The prognosis depends on the disease stage at diagnosis, and only localized carcinomas can be cured by radical treatment. Patients with localized prostate cancer could be divided into two groups on the basis of histological grade and tumor volume: those with poorly aggressive tumors that do not require treatment (given the patient's life expectancy) and those with larger, poorly differentiated tumors that carry a risk of dissemination and death. Unfortunately, it is not yet possible to do so in practice, since tumor volume cannot be measured accurately and the histological grade of biopsy specimens is not always representative of the whole tumor (prostate tumors are usually heterogeneous). Karyotype abnormalities have been detected in tumor cells, suggesting the existence of genetic alterations. These latter can be detected by methods based on cytogenetics and molecular biology. The identification of genetic alterations specific for prostate cancer would provide information on the mechanisms of tumor progression, and could serve as prognostic markers, like N-*myc* in neuroblastoma [26] and c-*erB-2/neu* [27] and *int-2* amplification [16] in breast cancer.

The steps involved in cell transformation, tumor formation and metastatic dissemination are poorly understood in prostate cancer. Tumor progression could be due to an accumulation of specific, consecutive genetic alterations with time, as is the case in colorectal cancer [12]. Results published by Carter et al. [6] favor a multistep process, and this would explain the increasing frequency of prostate cancer with age. In this scenario, the cell would acquire the ability to proliferate during the initial steps, and this proliferation would lead to further genetic alterations that account for invasive potential and metastatic spread. Candidate genes are those involved in

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physiological control of proliferation and differentiation (proto-oncogenes and tumor-suppressor genes), as well as those involved in proteolysis, cell adhesion and mobility [4], and DNA repair. Amplifications or mutation of proto-oncogenes are frequently found in various cancers; these events are known to activate proto-oncogenes. Indeed gene amplification is one mechanism that leads to overexpression. A limited number of studies concern prostate cancer, and we have focused on these areas.

Three proto-oncogenes, c-*myc*, c-*erbB-2* and *int-2*, are frequently amplified in adenocarcinomas of various origins, but particularly in breast cancer, which, like prostate cancer, is hormone dependent.

On the basis of the hypothesis that the progression of breast and prostate tumors might involve common factors, we used Southern blot analysis to detect amplification of these three proto-oncogenes in prostate tumors. The absence of significant amplification in most of the early "clinical" tumors we examined led us to evaluate 15 more-advanced tumors, 13 of which were stage C or D.

Table 1 Distribution of patients according to disease stage (Withmore-Jewett classification)

Stage	Patients (*n*)
B	2
C	7
D1	1
D2	5
Total	15

Table 2 Histological grade (Gleason classification)

Score	Patients (*n*)
2	1
4	3
5	2
6	4
7	1
8	4
Total	15

Materials and methods

The 15 tumor specimens were from patients with histologically proven prostate adenocarcinoma who had not previously received hormone therapy. The patients' mean age was 75 years (range 61–87 years). The Whitmore-Jewett disease stages and Gleason's tumor grades are shown in Table 1 and 2. Peripheral blood lymphocytes were used as a source of DNA for comparative analysis with the corresponding tumor, in order to detect somatic alterations of tumor DNA.

Procurement of prostate tumor specimens

Prostate tumor specimen were obtained in three ways, as follows:

Prostatectomy specimens

Five patient with "clinical" stage B carcinoma underwent radical prostatectomy with lymph node excision. In fact, histological examination of the surgical specimen and lymph nodes showed that one patient had pathologic stage D1 disease. A radical prostatectomy specimen was first sliced thickly and samples of tissue were cut out of suspect areas. One part of the specimen was fixed in formol for pathologic examination, while the other was immediately placed in liquid nitrogen for later molecular biology studies. The sample considered suitable for analysis if the proportion of tumor cells was 50% or more. Only three samples from the five patients were suitable for molecular biology studies (two contained only 5–10% of tumor cells). The three samples studied contained more than 80% of tumor cells.

Transurethral resection

The 11 patients who underwent this procedure all had "clinically advanced" tumors with local extension (clinical stage C) or bone metastases (stage D2). Between 6 and 12 chips were obtained during resection. Specimens were selected for DNA extraction as described above. Only 5 of the 11 patients' samples contained more than 50% of tumor cells and were thus suitable for analysis. The proportion of tumor cells was either 50% (three cases) or 70% (two cases). Three patients had stage C tumors and two had stage D2 tumors.

Needle biopsy

Samples were obtained in this way from 10 patients. The biopsy needle was introduced into suspect areas by the transrectal route using an automatic gun and a needle guide with digital control. Three 14-gauge biopsies (20 mm long and 1.8 mm wide) were taken from the same site, placed in liquid nitrogen and checked histologically. Samples were selected for DNA extraction as described above. The proportion of tumor cells varied between 50% (one case), 60–70% (four cases) and 80–90% (two cases). Three specimens were discarded, because they contained between 10% and 15% of tumor cells. The seven selected specimens were from patients who had advanced-stage disease with locoregional extension (four patients were in stage C); three had bone metastases.

Study of tumor and lymphocyte DNA by Southern blotting

Tumor DNA was extracted by incubating samples in lysis buffer containing proteinase K, then adding phenol-chloroform and precipitating with ethanol. Lymphocyte DNA was extracted by means of conventional methods [18]. DNA (10 µg) was digested with restriction enzymes specific for the probes used. After checking the quality of the digestion, the DNA fragments from each patient were separated by 0.8% agarose gel electrophoresis in the presence of ethidium bromide. After denaturation and neutralization of the gel, the DNA fragments were transferred to nylon Hybond N+ membranes (Amersham) in 20 × SSC (Salt Sodium Citrate) for 12 h. The membranes were then rinsed (2 × SSC) and dried, and the DNA was fixed by exposure to ultraviolet light.

The membranes were prehybridized with denatured calf thymus DNA then hybridized with specific probes labelled with ^{32}P by means of Nick translation (Amersham kit); oncogene amplifications were detected using the *pRyc 7.9* for c-*myc* gene [24], *SS6* for *int-2*/*FGF3* [7] and *pHER 2-436-1* for c-*erbB2*/*neu* [8]. The control probes were *pPL-8* for *CCND1* [20], *pHE 5.4* for *ETS1* [9] and *pHM2A* for c-*mos* (ATCC No. 41004).

The membranes were then washed in solutions containing 0.5% sodium dodecyl sulfate (SDS), as follows: 15 min at room temperature and then 15 min at 68 °C in 2 × SSC; twice in 0.2 × SSC at 68 °C

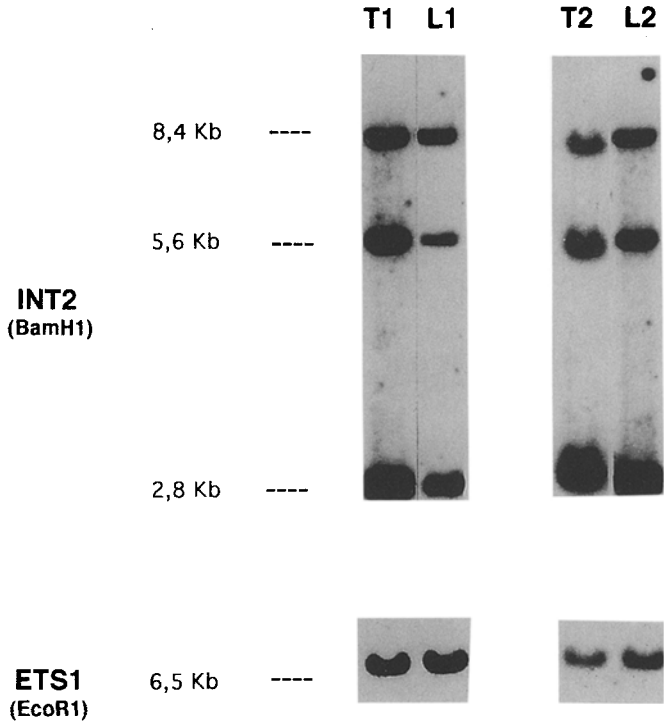

Fig. 1 DNA was digested with the appropriate restriction enzyme, then hybridized with a specific radioactive probe (*SS6* for *int-2*; *ETS1* as control probe). Tumor and lymphocyte DNA were migrated in parallel. *T1* and *T2* show tumor DNA from two patients, while *L1* and *L2* show lymphocyte DNA from the same patients. Both patients were heterozygous for *SS6*. Both alleles in tumor *T1* show amplification with the *SS6* probe, but not with the *ETS1* probe

for 15 min; and twice in 0.1 × SSC at 68 °C for 15 min. They were then placed in contact with Kodak film (X-omat K) at −80 °C for times that varied to the signal intensity.

Results

Oncogenes c-*myc* and c-*erbB-2/neu*

All the patients showed normal patterns of these two genes, regardless of the disease stage.

Oncogene *int-2*

We found one case of amplification in a heterozygous patient (Fig. 1), who had undergone a prostate biopsy which revealed 70% of tumor cells. The tumor was poorly differentiated (Gleason score 8), and the patient had stage D2 cancer with multiple bone metastases. This tumor also showed an amplification for the *PRAD1* probe located on the same amplicon. Moreover, no amplification was observed for *ETS1*, a more distal locus on chromosome 119. None of the other patients, including the other five who had metastases, showed *int-2* amplifications.

Discussion

The proto-oncogenes c-*myc*, c-*erbB-2* and *int-2* are frequently amplified in adenocarcinomas of various origins, but particularly in breast cancer, which, like prostate cancer, is hormone dependent.

The proto-oncogene c-*myc* encodes a protein that regulates DNA transcription by directly interacting with DNA. The gene(s) whose transcription is regulated by c-*myc* is (are) unknown. Abnormalities of this c-*myc* have been detected in various types of human tumors: c-*myc* is amplified or rearranged in about 30% of primary breast tumors [10], and in various proportions of ovarian, uterine, colon and stomach tumors [30]. C-*myc* is overexpressed in human prostate cancer [13], and this overexpression, detected by Northern blotting, correlates with the degree of differentiation [5]. In addition, c-*myc* is amplified and/or overexpressed in human prostate cancer cell lines [22, 23].

The proto-oncogene c-*erbB-2* (also known as *neu*) encodes a transmembrane phosphoprotein related to the EGF (epidermal growth factor) receptor, which has tyrosine kinase activity. Abnormalities of this gene and/or its expression have been detected in breast, ovary, colon and stomach cancers [30]. The prognosis of women with breast cancer correlates with c-*erbB-2* amplification [27]. This gene could also be involved in the progression of prostate tumors, as it is overexpressed both in tumors and tumor-derived cell lines [33]. However, in a recent study Mellon et al. [19] found no difference in the expression of c-*erbB-2* between locally advanced prostate tumors and prostate adenomas.

The proto-oncogene *int-2* has rarely been studied in patients with prostate cancer. Like the proto-oncogene *hst*, it belongs to the fibroblast growth factor (FGF) family; the two genes are situated in the same region (11q13) of the long arm of chromosome 11 [3]. *Int-2* and *hst* are situated on the same amplicon and are both amplified in 17% of primary breast tumors [1], as well as other types of cancer (upper respiratory tract and esophagus, malignant melanomas) [28, 30]. All the samples we studied contained a minimum of 50% of tumor cells, meaning that we were unlikely to miss a gene amplification because of an inadequate number of tumor cells.

The absence of c-*myc* and c-*erbB-2/neu* amplifications in the two localized stage B tumors is in agreement with the only previous study on such tumors reported in the literature (0/12 tumors according to Macoska) [17]. In addition, Macoska also described in detail the way in which sample were selected (50–80% of tumor cells), information that is crucial to the interpretation of the results.

The other 13 tumors analyzed here were from patients with extensive local invasion or distant metastases (stage C seven cases, stage D six cases). The absence of c-*myc* and c-*erbB-2/neu* amplification confirms the finding of Macoska on tumors with local extracapsular or lymph node extension (10 stage C tumors and stage D1 tumor). Although these genes do not appear to be involved in

tumor progression, even in advanced-stage prostate cancer with multiple bone metastases, these findings were obtained with a relatively small number of tumors.

In contrast, we detected an *int-2* amplification on a poorly differentiated tumor (Gleason score 8) in a patient with multiple metastases; to our knowledge, this has not previously been detected in human prostate cancer. The amplification seems to be limited to the 11q13 region since *prad-1* was co-amplified with *int-2*, whereas c-*ets* showed no amplification. *Int-2* is amplified in 14–16% of human breast tumors [16], and in head and neck tumors [28]. These amplifications we detected in the region 11q13 of DNA from an advanced tumor suggest that a gene in this region is involved in tumor progression; however, it remains to be determined if this amplification occurs in other cases. *Int-2* and *prad-1* amplifications are generally associated with amplification of two other genes, *hst* and *ems-1* located on the same amplicon [3]. *Hst*, like *int-2*, belongs to the fibroblast growth factor family and *ems-1* appears to be a cytoskeletal protein that is widely expressed in epithelial cells, whereas *prad-1* (also known as *cyclin D* and *CCND1*) encodes for a protein that shows structural homology with cyclins which are involved in cell cycle control [20].

The fact that *hst* and *int-2* are amplified but poorly expressed goes against an important role of either gene in prostate tumor phenotype. This suggests that the amplification unit contains one or several genes that may confer a selective advantage. *Int-2* is a marker of the amplicon but does not appear to be directly involved in carcinogenesis. *Ems-1* and *prad-1* are expressed at high levels in human tumors and cell lines in which they are amplified, contrary to *hst* and *int-2* [25]. Overproduction of the gene product could thus play a role in cell transformation by deregulating proliferation. However, it is not clear if either of these gene on the 11q13 region, frequently co-amplified, play significant role in the tumor. In addition, other genes can occasionally be altered, e.g., *p53* and the *ras* family genes. The latter tend to show mutations rather than amplifications. In the literature there are discrepancies concerning *ras* involvement in prostatic cancer, and the mutational frequency is between 0% and 32% [2, 21]. *Ras* onocogenes could be involved in metastasis formation through mutations [32] or overexpression [11]. Thompson has shown that cooperation between the oncogenes *ras* and *myc* can induce prostate cancer in a mouse model [31].

Conclusion

Further studies are required to determine the significance of the *int-2* amplification observed in this study, together with its frequency (particularly in metastatic disease). Needle biopsies allow a larger number of tumors of a given stage to be sampled, especially those which are not treated by radical prostatectomy or transurethral excision. Northern blot studies of *int-2* expression will show whether or not amplification is accompanied by overexpression. Alternatively, as in breast cancer, normally expressed *int-2* may be a simple marker of the amplicon located close to a co-amplified gene that is overexpressed and plays an important role in carcinogenesis. Clearly, no conclusions can be drawn as to the potential prognostic value of this isolated *int-2* amplification.

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