Distribution of CD44 messenger RNA in archival paraffin wax embedded tumours and normal tissues viewed by in situ hybridisation

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Abstract

Aims—We have previously demonstrated the abnormal localisation of expression of the CD44 gene in carcinoma cells in cryostat sections of fresh frozen tumour tissues, using radioactive in situ hybridisation (RISH). In order to facilitate further analysis of the expression of this gene in a wider range of neoplastic and non-neoplastic conditions, we have developed a technique which can visualise its low copy number transcripts in archival paraffin wax embedded specimens.

Methods—\(^1\)S labelled riboprobes complementary to transcripts from the standard (CD44s) and variant (CD44v) regions of the gene were used on paraffin wax embedded sections of tumours and corresponding normal tissues of the colon, breast and uterine cervix.

Results—Elevated levels of signals for CD44 and CD44v transcripts were observed in carcinoma cells relative to their non-neoplastic counterparts in all tissues examined.

Conclusion—This method permits easy access to material which can be selected for suitability, handled at room temperature without degradation and relied upon to show good histological detail. Comparison of the results with those on frozen tissues showed similar distributions of signals. Furthermore, the resolution and morphological detail was improved in paraffin wax sections.

(keywords: CD44, in situ hybridisation, tumour diagnosis)

The human CD44 gene encodes a family of cell surface glycoproteins which are involved in diverse intra- and extracellular functions. Abnormal expression of this locus has been observed in neoplasia in several organs and there is also evidence suggesting that it may play a role in tumour metastasis.\(^1\) The gene is composed of a stretch of approximately 60 kilobases on chromosome 11p, comprising at least 21 exons. Ten of these (that is, exons 1–5 and 16–20) are expressed together on all cell types as the standard form (CD44s) and the remaining 11 can be included by alternative splicing to generate a number of variant isoforms (CD44v).\(^2\) The regulation of expression of the CD44v isoforms is unknown but data from several laboratories now confirm that the overproduction of protein isoforms and of many transcripts, including immature cytoplasmic mRNA species containing introns, is associated with the onset and progression of tumours in many organs.\(^3\)

This information has been obtained by a combination of techniques including immunohistochemistry\(^4\) and reverse transcription followed by the polymerase chain reaction (RT-PCR)\(^5\)\(^\_\)\(^6\). However, although the former gives information regarding the distribution of CD44 proteins and the latter gives an insight into differences in relative levels of the transcription of the gene in normal and tumour tissues, neither can be used to view the cellular distribution of CD44 transcripts. The distribution of proteins observed by immunohistochemistry does not necessarily reflect gene transcription and the RT-PCR technique requires homogenisation of the tissue. Therefore, in situ hybridisation used in conjunction with the other techniques can provide valuable extra information on the disordered expression of this gene in neoplasia.

We have previously reported observations obtained with radioactive in situ hybridisation (RISH), on CD44 expression in fresh frozen colonic tumours and their normal tissue counterparts.\(^7\) That study demonstrated the power and value of such integrated investigations, by showing that the increased expression observed in heterogeneous tumour tissues resulted from increased transcription in the tumour cells and that it can be seen in early lesions, including adenomas. In order to extend such work it was deemed important by us to circumvent the problems of working with frozen tissue samples, such as limitation of material available from suitable clinical cases and rapid degradation of mRNA if the specimen thaws. We have therefore developed the following method for use on fixed, paraffin wax embedded tissues, which liberates the investigator to use specifically selected specimens from routine pathology service archives and to conduct the bench work at room temperature, at their convenience. We knew from other evidence\(^8\) that the copy number of CD44 gene transcripts is relatively low, compared with housekeeping genes, even in tumour tissue and so we used RISH in order to maximise the sensitivity of the methodology.

Methods

Paraffin wax blocks containing tumour tissue and adjacent non-neoplastic tissue were selected from the archives. For this study we used...
single blocks from five separate cases of colonic carcinoma, four from breast carcinomas and two from carcinomas of the uterine cervix. Four further blocks from normal cervical tissue were also studied.

**IN SITU HYBRIDISATION**

In situ hybridisation was carried out using a modification of the method described by Simmons et al., using single stranded $^3$S labelled RNA probes. Paraffin wax sections, 8 μm thick, were dewaxed, rinsed in ethanol, rehydrated through a graded series of ethanols, made in 0.9% NaCl and finally rinsed in phosphate buffered saline (PBS). Tissue was post-fixed in 4% paraformaldehyde (in PBS) for 20 minutes, rinsed in PBS twice and digested with 10 mg/ml proteinase K (Boehringer Mannheim) for eight minutes. Tissues were again post-fixed in 4% paraformaldehyde (in PBS) for five minutes, rinsed in PBS 0.1 M triethanol amine (TEA) and acetylated in 0.25% acetic anhydride for 10 minutes at room temperature. Slides were finally rinsed in 2 × SSC and dehydrated in a graded series of ethanol solutions. Sections were dried for at least two hours before being hybridised overnight in a solution of 50% formamide, 10% dextran sulphate, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 × Denhardt’s solution, 0.5 mg/ml tRNA and 10 mM DTT, with 1 × $10^4$ cpm/ml $^3$S labelled single stranded RNA probe at 50–60°C. Sections were washed in 4 × SSC, digested with 20 mg/ml RNase A at 37°C for 30 minutes, then washed at a final stringency of 0.1 × SSC at 60°C for 30 minutes and dehydrated in a series of ethanol and dried. Slides were hand-dipped in Kodak NTB-2 emulsion and then exposed at 4°C for two weeks. After developing, sections were stained with haematoxylin and eosin, mounted and photographed.

**PREPARATION OF RNA PROBES FOR IN SITU HYBRIDISATION**

$^3$S labelled riboprobes for CD44 standard and variant transcript detection were synthesised as described previously.

**Results**

The observations described below were reproducible in all specimens in each category.

**COLONIC TISSUES**

The intensities of the signals obtained using the riboprobes for CD44s and CD44v were much greater over the tumour tissue than over the adjacent non-neoplastic mucosa. Within the tumours, the CD44s mRNA riboprobe was localised in much greater abundance over the carcinoma cells (figs 1A and 1B) than over the fibroblasts and inflammatory cells in the intervening stroma. In the normal mucosa this probe reacted weakly with epithelial cells at the bases of the crypts. Epithelium elsewhere was negative, although CD44s transcripts were seen in most of the stromal cells in the lamina propria. CD44v transcripts were almost undetectable in normal colonic epithelium but in all the tumours very high levels were seen specifically in the carcinoma cells (figs 1C and 1D) and not in other cell types. Hence, the results...
obtained with paraffin wax sections were similar to those obtained previously with cryostat sections of fresh frozen colonic tissue.9

BREAST TISSUES
In all the normal breast tissue specimens, weak signals for both CD44s and CD44v could be seen over the luminal epithelial cells as well as over the myoepithelial cells. The CD44s probe also hybridised to the cells in the surrounding stroma but the one for CD44v did not do so above background level (figs 2A and 2B). In contrast, both probes showed marked elevation of signal intensity over the carcinoma cells in these sections but the one for CD44v hybridised only with carcinoma cells and not with stromal cells in all tumours studied. This therefore provided a clear demonstration that the abnormally high levels of alternatively spliced CD44v gene transcripts detected previously by RT-PCR in tumours are located specifically in the carcinoma cells.

TISSUES FROM THE CERVIX UTERI
In normal cervical squamous epithelium both standard and alternatively spliced CD44 transcripts were most prevalent in the basal layer and declined substantially in the adjacent overlying layers (figs 2C and 2D). Interestingly, the CD44v signal was significantly greater than that of CD44s over this type of epithelium. In the specimens of cervical carcinomas this technique indicated that the transcripts for both CD44s and CD44v were increased in the carcinoma cells relative to others. The intensity of the CD44v signal was higher than that of CD44s over the malignant epithelium and it was absent over the stromal and inflammatory cells (figs 2E and 2F).

Discussion
This work demonstrates that changes which occur in CD44 transcription during neoplasia in several organs can be visualised reproducibly in fixed, paraffin wax embedded, archival

Figure 2 Paraffin wax sections of breast carcinoma hybridised with antisense (A) CD44s and (B) CD44v riboprobes. Normal ectocervical epithelium hybridised with antisense (C) CD44s and (D) CD44v riboprobes, showing a decrease in signal towards the epithelial surface. Sections of a cervical carcinoma hybridised with (E) CD44s and (F) CD44v antisense riboprobes. (Original magnification, ×360.)
pathological specimens. As in the previous study of frozen tissue, it was shown that the changes are localised within the carcinoma cells. With this method it now becomes possible to conduct correlated investigations on adjacent histological sections from specifically selected archival tissue specimens to analyse relations between CD44 gene transcription and translation in pre-neoplastic lesions and during tumour progression. From such work it is hoped to build a better understanding of when the marked abnormalities in CD44 gene expression in neoplasia begin, how they inter-relate with each other and whether any of them could be used as a simple diagnostic or prognostic tool in clinical practice. The application of such techniques to archival material will be particularly suitable for the investigation of pathological conditions which are in themselves believed to be non-neoplastic—for example, atypical ductal hyperplasia of the breast, but which signal an increased risk of developing invasive malignancy at some future date. These lesions, being microscopic and impalpable, are discovered as incidental findings in specimens resected for other purposes and it is therefore advantageous to study them in selected specimens, chosen from the departmental archives. At present, there is no way to identify which of the patients found to have these risk related lesions will later develop malignant tumours. It is hoped that the ability to investigate conveniently the status of transcription and translation of CD44 and other genes in histological sections may uncover a marker or a group of markers which could be helpful in the evaluation of prognosis in patients with such lesions.

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