

Surface Protein Expression and Messenger RNA-splicing Analysis of CD44 in Uterine Cervical Cancer and Normal Cervical Epithelium¹

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Abstract

Variant CD44 has recently been shown to serve as a metastasis marker in human breast cancer. Certain variant epitopes on primary tumors predict poor survival probabilities for the patients. In this study, immunohistochemical analysis of 16 uterine cervical carcinomas showed strong expression of several CD44 variant epitopes in all samples. In normal cervical epithelia from 5 patients, expression of these epitopes was restricted to particular cell layers, with expression being strong in basal and spinal cells but absent in superficial cells. Fifteen of 16 cancer samples were stained strongly with an antibody which recognizes one particular CD44 epitope that is encoded by both variant exons v7 and v8. This epitope was not detectable in normal cervical epithelium. CD44-mRNA splicing analysis showed qualitative and quantitative differences between malignant and normal tissues with a much more complex splice pattern and high expression of a large CD44 isoform containing variant exons v3 to v10 (including the v7/v8 transition epitope) in about one-half of the cancer samples. Interestingly, patients with lymph node metastases were in this group only. These differences in CD44 epitope expression and mRNA splicing in cervical carcinoma reveal dynamic changes in CD44 expression during carcinogenesis. Such changes could provide metastatic cells with a selective advantage during the carcinogenic process. Furthermore, the v7/v8 epitope may be suitable for screening early stages of cervical cancer.

Introduction

Despite decreasing incidence rates (1), the prognosis for patients suffering from advanced stages of cervical cancer is poor (2-4). Early diagnosis is based on the pathological evaluation of early morphological changes in the epithelial cells (cervical smear). It would be desirable to find defined molecular markers for early cancer detection which can be used for staging and as prognostic factors. Expression of alternatively spliced CD44 isoforms, carrying the so-called variant exons v5 and v6, correlates with advanced stages of human breast and colon cancers (5)³ and aggressive and metastatic human lymphoma (6) and is found on metastatic rat pancreas and breast cancer cell lines (7). Several laboratories have also reported changes in CD44 splice patterns in human cancers (8, 9). Experimental animal studies and clinical evaluation both strongly suggest causal roles for these proteins in tumor progression and particularly in lymphogenic metastatic spread.

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The normal functions of variants of CD44 are, however, unknown. Many epithelia carry splice variants, including the epithelial cells of human skin, bladder, stomach, and uterine cervix (7, 10). It has not yet been investigated whether cancer cells originating from such epithelia make use of these or other splice variants of CD44 during metastatic disease. We show here that normal cervical epithelium expresses variant epitopes. During carcinogenesis, the pattern is changed: CD44v⁴ synthesis is enhanced overall and a new epitope appears which is reflected by new RNA splice variants.

Materials and Methods

Tumor and Normal Tissue Samples. Cancer samples and normal tissue samples of human uterine cervix were provided by the Department of Gynecology, University of Heidelberg (Germany). All samples had been snap-frozen in liquid nitrogen immediately after surgical excision and stored at -80°C until usage. Five normal tissues were obtained from patients undergoing a total hysterectomy due to nonneoplastic diseases. Sixteen cervical cancer samples (15 squamous cell carcinomas and 1 adenocarcinoma) were also obtained. The stage of the tumors ranged from International Federation of Gynecologists and Obstetricians stage Ib ($n = 7$) and stage IIb ($n = 5$) to stage IIIb ($n = 4$).

Immunohistochemistry and Antibodies. All deep-frozen tissue samples were cut into 7- μ m-thick tissue layers and fixed. Immunostaining was performed as described previously using standard protocols (5, 8). Monoclonal antibodies (mouse anti-human mAb) and polyclonal sera (rabbit anti-human antiserum) directed against different variant epitopes of the CD44 molecule were used (Fig. 1), the specificity of which was shown elsewhere (5, 6). The mAb VFF17 is directed against an epitope formed jointly by exons v7 and v8. The mAb SFF2 recognizes an epitope close to the NH₂ terminus of the CD44 standard portion staining CD44s as well as CD44v. Either biotinylated anti-mouse (mAbs) or anti-rabbit (polyclonal sera) F(ab')₂ (DAKO Corp., Santa Barbara, CA) were used as secondary antibodies for the streptavidin-biotin-peroxidase complex (DAKO Corp.). After the cell nuclei were counterstained with hematoxylin, the sections were mounted with glycerol-gelatin and were then evaluated.

Reverse Transcription-PCR Amplification. Three μ g of total RNA (isolated from tumor and normal tissues) were prepared and reverse-transcribed as described previously (7). Five μ l of first-strand cDNA were amplified by Taq polymerase (Amersham) in a volume of 50 μ l, using Taq polymerase buffer (Amersham). For glyceraldehyde-3-phosphate dehydrogenase PCR oligonucleotides homologous to positions 8-29 and 362-339 of the published cDNA sequence (11) were used. For amplification of variant CD44 transcripts, primers homologous to positions 513-540 and 934-958 of the published human CD44 sequence (12) were used. After 25 (glyceraldehyde-3-phosphate dehydrogenase) and 30 (CD44) cycles of amplification (94°C for 1 min, 62°C for 1 min, 72°C for 2.5 min) 10 μ l of the reaction mixture were resolved on 1.0% agarose. The amplification products were visualized under UV light after ethidium bromide staining and the CD44 products were subsequently transferred onto nylon membranes (Hybond N+, Amersham) for Southern blotting. ³²P-Labeled hybridization probes were synthesized by PCR using variant

⁴ The abbreviations used are: CD44v, variant CD44; mAb, monoclonal antibody; CD44s, standard CD44; cDNA, complementary DNA; PCR, polymerase chain reaction.

CD44 exon-specific primers homologous to positions 24–53/81–110 (v3), 128–154/213–239 (v4), 243–271/327–356 (v5), 357–383/456–482 (v6), 489–515/585–614 (v7), 621–647/692–718 (v8), 722–750/779–808 (v9) and 812–838/987–1013 (v10) (13).

Results and Discussion

Sixteen cervical carcinomas and 5 samples of normal cervical epithelium were screened for the surface expression of NH₂-terminal and of variant exon epitopes of CD44 (Fig. 1), and for CD44-mRNA by reverse transcriptase-PCR (see "Materials and Methods"). These data permit conclusions for both (a) normal cervical differentiation and (b) carcinogenesis.

Epithelial Differentiation. NH₂-terminal epitopes are present on subepithelial stromal cells and throughout all epithelial layers (Fig. 2G). The presence of NH₂-terminal epitopes indicates CD44 promoter activity in all stromal cells and epithelial layers. Epitopes of variant exons are absent from stromal cells but are strongly expressed by epithelial cells. However, the epitopes are lost toward the epithelial surface and are absent from the stratum superficiale (Fig. 2, A, C). One epitope formed by the transition part of exons v7 and v8 recognized by the mAb VFF17 (anti-v7/v8) is totally absent from normal epithelium (Fig. 2E). This pattern of immune staining could be interpreted to indicate the surface expression of one or several CD44 isoforms with regulated accessibility of epitopes (e.g., by glycosylation) or the regulated synthesis by alternative splicing of several isoforms, thereby excluding the exon v7/v8 combination indicated by the transition epitope. The O-glycosylation site close to the 5'-end of exon v8 (7, 14) could result in nonaccessibility of the epitope to the antibody. Regulated synthesis of variant exon containing CD44 isoforms has been reported previously in activated lymphocytes and in skin keratinocytes (15, 16). In the cervical epithelium the highly active and strongly proliferating basal cells show strongest CD44v expression whereas the more quiescent superficial cells

do not, although promoter activity, shown by the NH₂-terminal epitope, exists. The differences, therefore, represent changes in splicing or glycosylation.

To explore whether alternative splicing can be detected, RNAs were analyzed by semiquantitative reverse transcriptase-PCR. Long-exposure hybridization with variant exon probes (synthesized by PCR with exon-specific primers) reveals the presence of alternatively spliced longer CD44 transcripts in the RNA samples (Fig. 3). The largest band detected by all probes, around 850 base pairs (Fig. 3, arrows), could carry 3 to 4 variant exon sequences. Due to hybridization with all variant exon probes, this band must represent a mixture of different mRNA species. One of these mRNAs very likely represents the epithelial variant (v8-v10) which exhibits a theoretical length of 842 base pairs (446 base pairs for CD44s plus 396 base pairs). The data suggest but do not prove that a CD44 isoform that contains v7 and v8 at the same time is not expressed. This would be in agreement with immunohistochemistry, by which we cannot detect the transition (v7/v8) epitope. The source of CD44s RNA cannot unequivocally be defined. Whether the stratum superficiale synthesizes only the CD44s isoform or modifies variant epitopes cannot be determined from the existing data.

Carcinogenesis. In distinct contrast to normal epithelium, 15 of 16 samples of cervical cancer carry the v7/v8 transition epitope (Fig. 2F). This indicates that there is either a drastic change in the modification of epitopes or, more likely, a drastic change in the splice pattern. Furthermore, although immunostaining is only semiquantitative, antibody dilution experiments have suggested that CD44 expression on all cancer cells is enhanced over normal epithelium. PCR data show that almost all samples of malignant tissue carry RNA for CD44 splice variants (Fig. 4). Fig. 4, Lanes 6 and 9, represents samples with only a small number of tumor cells. Therefore, extractable CD44v RNA was too little to be sufficiently amplified. The tumor cells, however, showed strong variant epitope expression in the immune staining. The

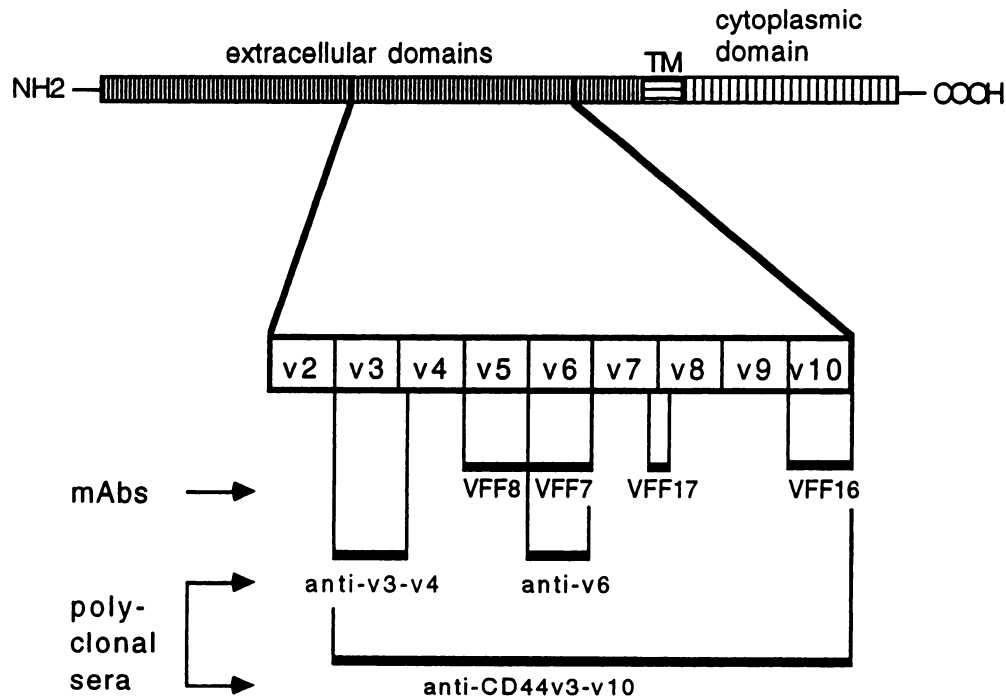


Fig. 1. Schematic representation of a CD44 splice variant. This putative variant carries the variant exons v2 to v10. The variant region is inserted within the extracellular domain close to the transmembrane region (TM). The polyclonal sera anti-CD44v3-v10, anti-v3-v4, and anti-v6-v7 react with bacterial fusion proteins encoded by variant exons v3-v10, v3-v4, and v6-v7, respectively. The other columns indicate the approximate locations of the epitopes for the monoclonal antibodies VFF7, VFF8, VFF16, and VFF17. The antibodies VFF7, VFF8, and VFF16 are presumably specific for one exon, whereas mAb VFF17 reacts with an epitope jointly encoded by exons v7 and v8.

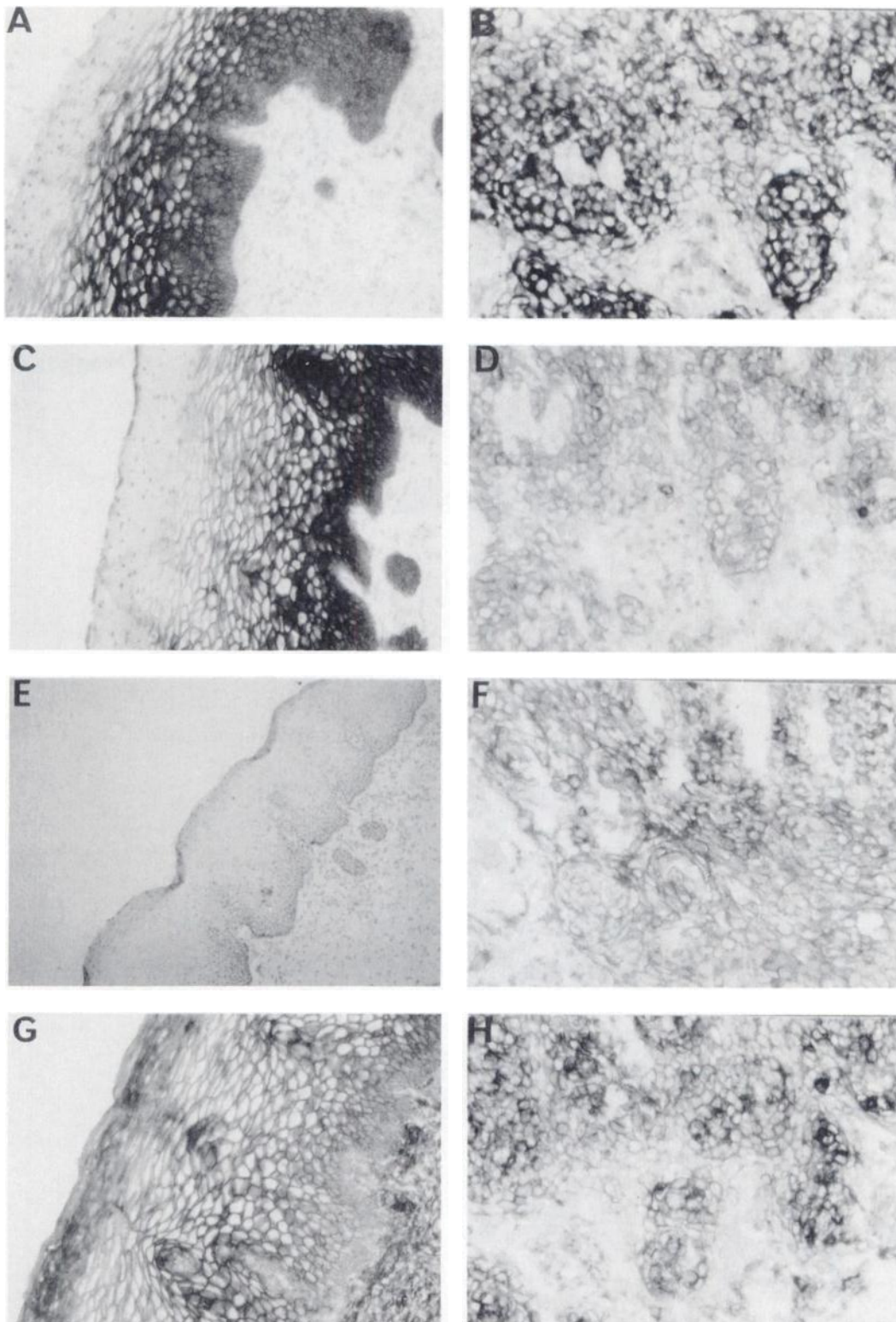


Fig. 2. Immunohistochemistry of normal uterine cervical epithelium (A, C, E, G) and cervical cancer (B, D, F, H). All normal cervical tissue samples show a strong staining reaction with the polyclonal sera anti-CD44v3-v10 (A), anti-v3-v4 (C), anti-v6-v7, VFF7, VFF8, and VFF16 (not shown). The staining reactions were limited to the stratum basale and the stratum spinosum, while the stratum superficiale remained unstained. The mAb VFF17 showed no staining of normal epithelial cells (E). The mAb SFF2, directed against CD44s, stained strongly all cell layers. Most of the cervical cancer samples showed strong staining reactions with all polyclonal sera and mAbs, including mAb VFF17. Representative examples are shown in Fig. 2, B (anti-CD44v3-v10), D (mAb VFF7), F (VFF17), and H (mAb SFF2). Avidin-biotin-peroxidase complex method. A-D, F-H, $\times 140$; E, $\times 80$; counterstain, hematoxylin.

ethidium bromide stain documents the relative abundance of PCR products in tumor samples as compared to that of normal epithelium (exposure difference 20-fold) (Fig. 3). Note, however, that the band corresponding to CD44s may originate from stromal cells. The rela-

tive abundance of variant exon sequences differed from one sample to the next. Interestingly, the probes with most abundant expression included those from patients that at the time of surgery already had signs of lymph node metastases.

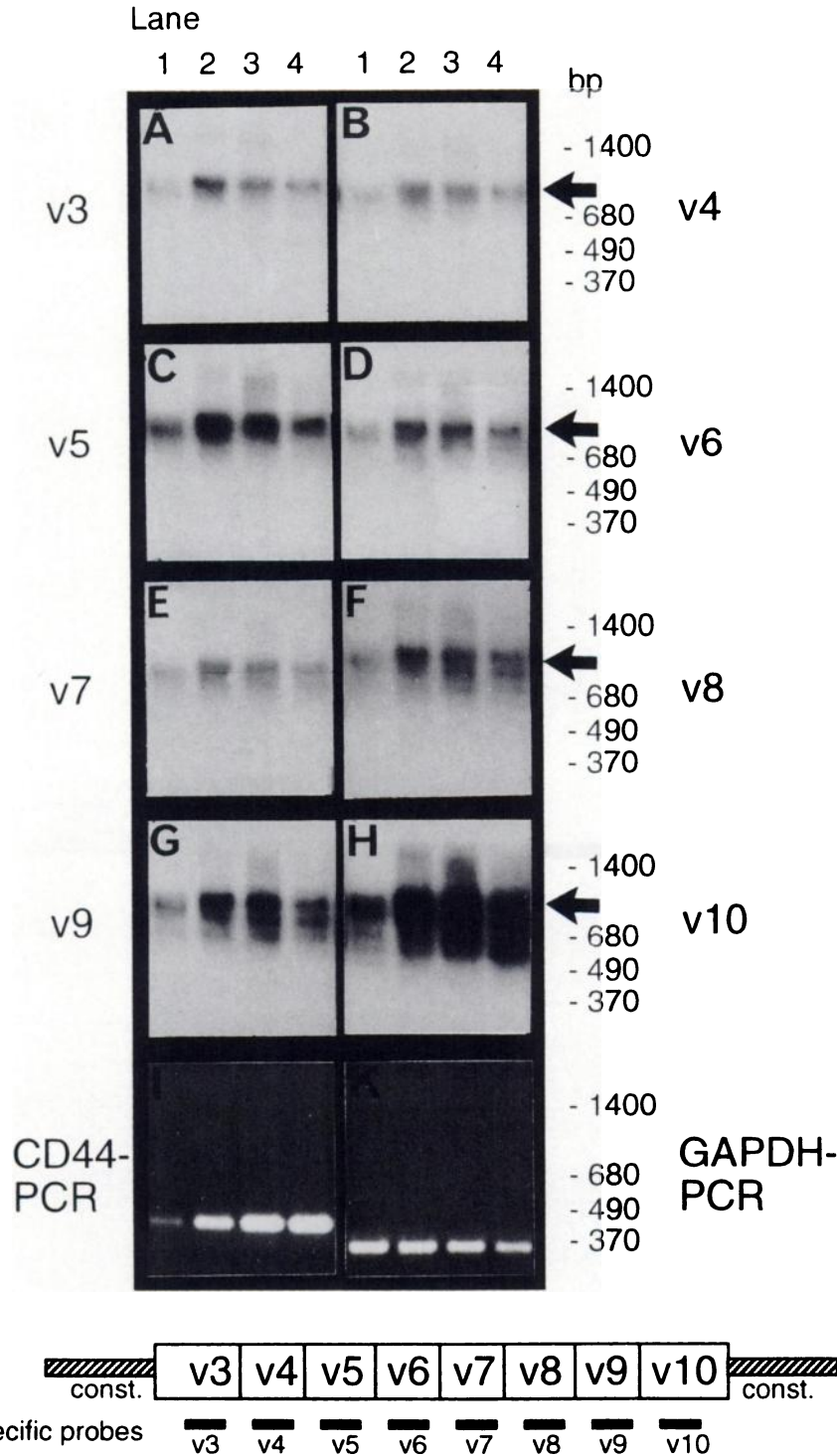


Fig. 3. Southern blot analysis of PCR amplification products from individual samples of normal uterine cervical tissues. The PCR primers were specific for CD44 exons adjacent to the variant exon sequences. cDNAs were produced by reverse transcription and were tested by glyceraldehyde phosphate dehydrogenase PCR prior to CD44s amplification, to check for quality and abundance of the cDNAs synthesized (K). The PCR products obtained with CD44 standard primers (see "Materials and Methods") were resolved on 1.0% agarose (J) and transferred to a Hybond N+ membrane (Amersham), and the same filter was hybridized consecutively to probes specific for the variant exons v3-v10 (A-H) as indicated in the figure. Lanes 1-4, normal cervical samples of four different patients. Exposure time, 45-60 min.

A large mRNA species (1440 base pairs; see arrows in Fig. 4) compatible with an isoform of CD44 with exons v3 to v10 was frequently produced in the cervical carcinomas tested in this study. This could be the isoform containing the v7/v8 transition epitope. In addition, several smaller isoforms are detectable, perhaps (from the hybridization pattern) CD44 v3 to v7 and CD44 v8 to v10, as well as other variants.

In conclusion, cervical carcinomas appear to demonstrate a 2-fold change in CD44 expression: enhancement of production and surface exposure of CD44 splice variants; and a change of splice pattern leading to the acquisition of a new (transition) epitope. Although it is not clear what molecular functions individual splice variants exert, the change in splice pattern appears to convey selective advantage. Further investigations on different stages of cervical dysplasias will be

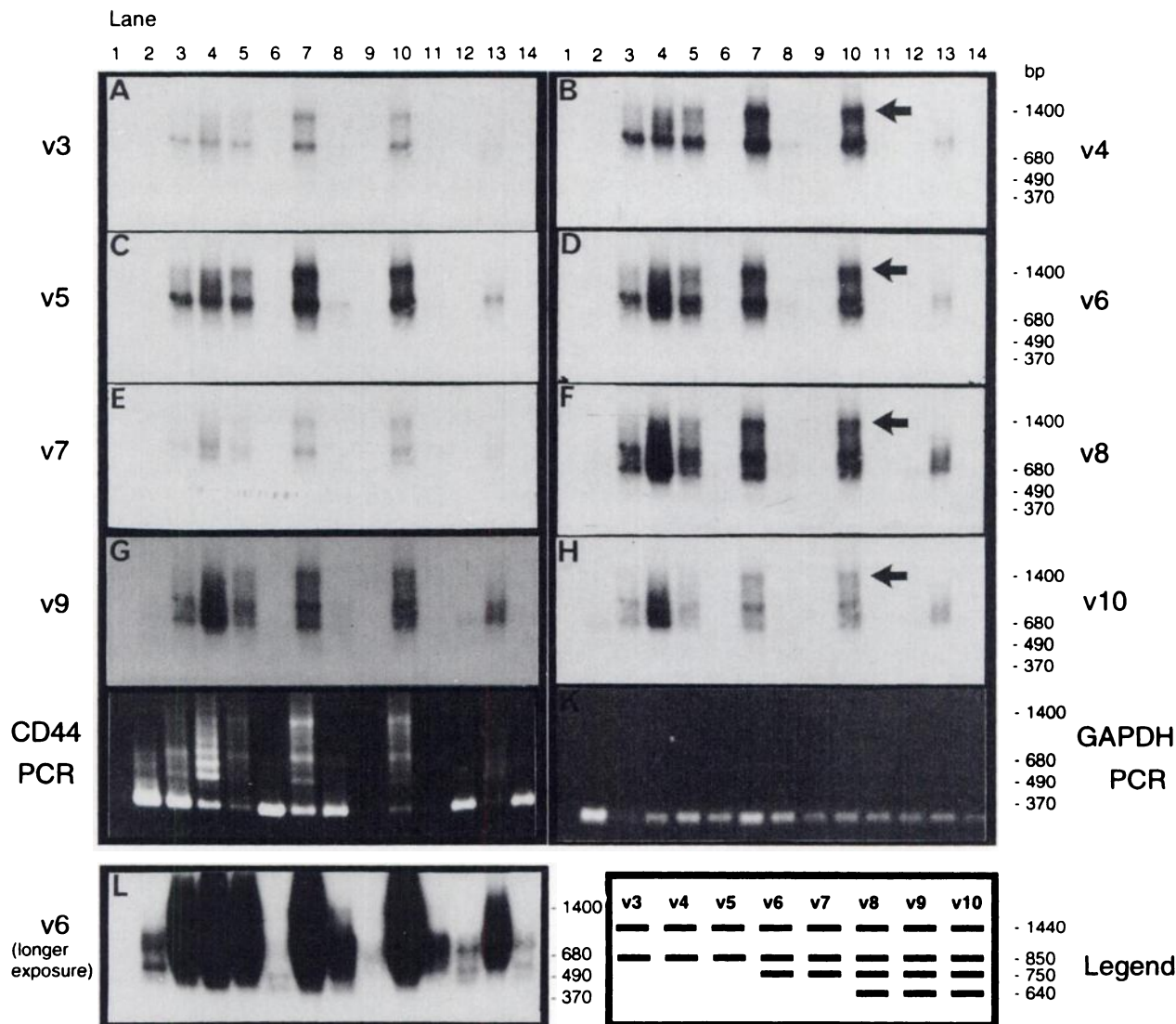


Fig. 4. Southern blot analysis of PCR amplification products from individual samples of uterine cervical cancer. The PCR primers were the same as described in Fig. 3. The PCR products obtained with CD44 standard primers (see "Materials and Methods") were resolved on an agarose gel (I), and after Southern blotting the filter was hybridized consecutively to probes specific for the variant exons v3-v10 (A-H) as indicated on the figure. Lane 1, negative control; Lane 2, breast cancer sample as positive control; Lanes 3-14, cervical cancer samples of 12 different patients; Lane 7, same tumor as shown in Fig. 2. Exposure time, 2-5 min (A-H), 15-20 min (L).

performed to determine when during carcinogenesis the change in v7/v8 epitope expression occurs and to study its possible role as an early cancer detection marker.

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