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## Differential expression of ferritin Heavy chain in a rat transitional cell carcinoma progression model

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### Abstract

To identify molecular markers with predictive value for the progression of superficial bladder cancer we used the differential hybridization analysis approach. Since primary tumor material is heterogeneously composed of subpopulations that are poorly characterized, we used in this study a rat progression model system that phenotypically and cytogenetically resembles human superficial bladder cancer. In the differential hybridization analysis we compared the mRNA populations of low and high metastatic tumor lines. We observed an overexpression of ferritin Heavy chain (ferritin H) in the tumor line with the lower metastatic capacity and better differentiated phenotype. The exact clinical relevance for the differential expression of ferritin H in human bladder cancer remains to be determined.

*Keywords:* Differential hybridization analysis; Transitional cell carcinoma; Ferritin H chain

### 1. Introduction

An important issue in the management of superficial transitional cell carcinoma (TCC) of the bladder is to identify those patients that are at risk for progression to invasive disease. Since primary tumor material is usually heterogeneously composed of subpopulations that are poorly characterized, it is not suitable to identify TCC progression markers. The identification of such parameters requires a tumor model system in which the different stages of tumor progression are represented.

In this study a rat bladder tumor model system (RBT) is used that phenotypically and cytogenetically resembles human superficial TCC [1]. Two spontaneously arisen bladder tumors (in ACI rats) RBT 323 and RBT 157, were serially transplanted. In the fifth transplant generation the RBT 323 becomes metastatic to the lungs in more than 90% of animals. The metastatic ability of the RBT157 tumor changes from low to intermediate (50% of the rat have lung metastases) in the fourth passage. The tumor doubling time of the RBT 323 tumor decreased from 13 days to 3.5–4 days, while the tumor doubling time of the RBT 157 tumor remained almost unchanged. Both tumor lines initially resembled grade II TCC. However, while the histological pattern of the RBT 157 remained essentially unchanged, the RBT 323 tumor progressed to a grade III tumor in the third passage [1]. In order to identify genes that are associated with

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the progression of superficial bladder cancer we applied the technique of differential hybridization analysis to compare the mRNA levels of the RBT 323 with the RBT 157 line, two lines that differ in their metastatic capacity. The cDNA clones that detected differentially expressed genes were further evaluated for their relationship to the metastatic phenotype by means of Northern blot analyses. The cDNA clones of interest were sequenced and the resulting nucleotide sequences were compared to nucleotide database to search for homology with known genes.

## 2. Materials and methods

### 2.1. Tumor specimens

Tumor material from a rat bladder tumor model system (RBT) [1] was used in this study (Table 1). Transplant passage #10 of the highly metastatic line RBT 323 and passage #1 of the moderate metastatic line RBT 157 were used for the differential hy-

bridization experiments. In order to check the relevance of the selected clones passages 1, 2, 8, 9, 15 and 16 of the RBT 323 line and passage 1 and 6 of the RBT 157 line were used.

### 2.2. Construction of cDNA libraries

A directionally cloned cDNA library was constructed using polyadenylated cytoplasmic RNA from the highly metastatic RBT323 line, passage #10. Poly(A<sup>+</sup>)RNA (5  $\mu$ g) was fractionated from total RNA by oligo(dT)-cellulose chromatography and total RNA was isolated using the lithium chloride/urea procedure [2]. First strand cDNA was synthesized by using an oligo(dT) primer-adaptor (Promega) containing a NOT-1 site 5' to the oligo(dT) tail. Second strand cDNA synthesis was performed using the procedures described by Gubler and Hofman [3]. The cDNA was blunt ended by using T4-DNA polymerase (10 U). After purification using micro colloid bags (Sartorius GmbH), *Eco*R1 linkers (1  $\mu$ g, Stratagene) were ligated to the blunt ended cDNA by means of T4-DNA ligase, followed by digestion with NOT-1 and *Eco*R1 restriction endonucleases. The digested linkers were separated from the cDNA by means of PurElute™ agarose gel electrophoresis (Invitrogen) and the cDNAs with the desired size range (0.5–10 kb) were isolated from the agarose. Electroelution of the cDNA from the agarose was performed using the Geneluter™ electroelution system (Invitrogen). Subsequently the ds cDNA was ligated in the Lambda Zap-II cloning vector (Stratagene), inserted into the phage particles using Promega Packagene Lambda DNA Packaging System and transformed to XL-1 Blue *E. coli* bacteria.

### 2.3. Differential screening of the cDNA library

For the differential screening of the RBT 323/10 cDNA library, 36 000 recombinant phages were plated. After overnight incubation, 4 replicas of each plate were taken on nitrocellulose by incubation during 1, 3, 10 and 30 min, respectively. The filters were next lysed according to Sambrook et al. [4]. Hybridization was performed according to Hanahan and Meselson [5] in 40% formamide at 42°C during 60 hours. For the differential screening, probes representative for the RBT 323/pass · 10 and RBT 157/pass

Table 1  
Growth characteristics of rat bladder tumor lines

| Tumor passage | Tumor doubling time <sup>a</sup> | Metastases <sup>b</sup> |
|---------------|----------------------------------|-------------------------|
| RBT323        | ND                               | 0/1                     |
| Passage 1     | ND                               | 0/6                     |
| Passage 2     | 13.4 (1.4)                       | 0/8                     |
| Passage 3     | 11.8 (1.2)                       | 1/10                    |
| Passage 4     | 5.9 (1.0)                        | 0/9                     |
| Passage 5     | 5.7 (0.3)                        | 3/7                     |
| Passage 6     | 4.4 (0.1)                        | 11/13                   |
| Passage 8     | 3.8 (0.2)                        | 15/15                   |
| Passage 10    | 3.7 (0.2)                        | 9/9                     |
| Passage 12    | 4.2 (0.3)                        | 19/22                   |
| Passage 13    | 3.3 (0.3)                        | 6/6                     |
| Passage 15    | 4.3 (0.3)                        | 5/6                     |
| Passage 16    | 4.0 (0.3)                        | 6/6                     |
| RBT157        | ND                               | 0/1                     |
| Passage 1     | 11.2 (0.8)                       | 0/4                     |
| Passage 2     | ND                               | 1/7                     |
| Passage 3     | 10.5 (1.1)                       | 2/9                     |
| Passage 4     | 8.2 (0.3)                        | 5/8                     |
| Passage 6     | 9.5 (1.8)                        | 2/6                     |

<sup>a</sup> Tumor doubling time in days, SEM in brackets (ND, not determined).

<sup>b</sup> Number of rats with lung metastases/total number of rats implanted with respective tumor passage.

• 1 mRNA populations were prepared by random prime labeling [6] of first strand cDNA. For hybridization of the filters,  $1 \cdot 10^6$  dpm/ml hybridization solution was used. The replicas 1 and 10 min were hybridized with probe derived from the RBT 157/pass.1 and the replicas 3 and 30 min were hybridized with probe derived from the RBT 323/pass.10. The blots were washed according to Hanahan and Meselson [5]. The filters were exposed for autoradiography during three days. Extensive comparison of the autoradiograms resulted in selection of differentially expressed clones.

#### 2.4. Phagemid isolation

Putative differential expressed cDNA clones were purified and phagemids were isolated according to the Stratagene protocol.

Sequence analysis was performed with the Sequenase Version 2.0 System (United States Biochemical). M13 and reverse primers were used allowing sequence analysis from both ends of the clones. Com-

#### 2.6. Sequence analysis and computer analysis

For the northern blot analysis,  $10 \mu\text{g}$  of total RNA was glyoxylated, size fractionated on 1% agarose gels and transferred to Hybond-N<sup>+</sup> nylon membranes (Amersham). Probes were made of the differentially expressed cDNA clones, obtained from small scale plasmid isolations or of purified inserts of the cDNA clones, by random prime labeling reactions [6]. Hybridization experiments were performed according to Church and Gilbert [7].

#### 2.5. Northern blot analysis

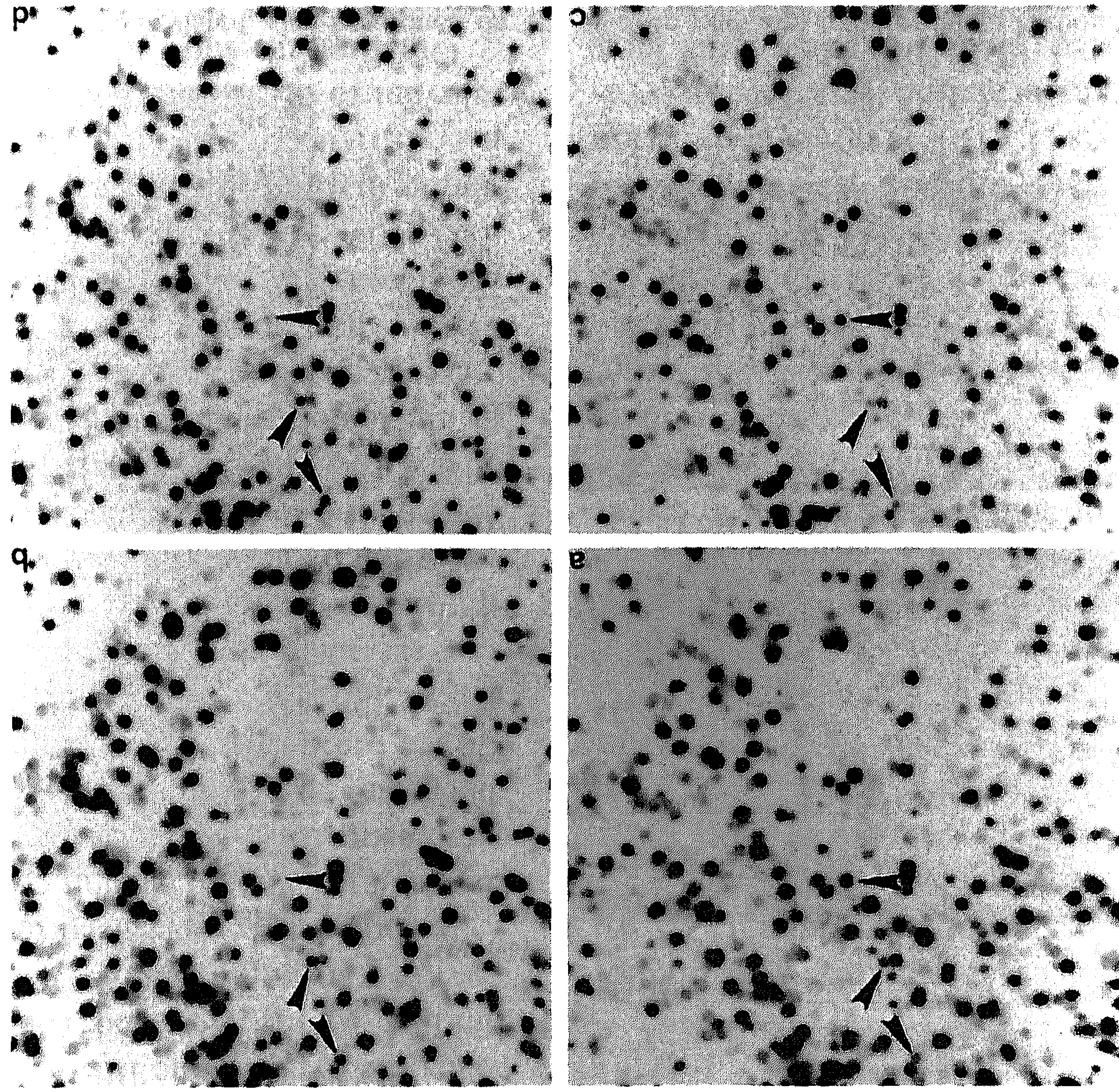


Fig. 1. Differential screening of the RBT 323/10 cDNA library. Replicas were hybridized to cDNA probes prepared using poly-A<sup>+</sup>-RNA from the RBT 157/1 line (a + c) or from the RBT 323/10 line (b + d). The arrows indicate the three most significant differentially expressed cDNA clones.

puter assisted comparison studies of the sequences were performed with the EMBL and Genbank nucleotide sequence databases.

### 3. Results

#### 3.1. Differential screening

A cDNA library was constructed from the mRNA of the highly metastatic line RBT 323/passage 10. The complexity of the library was  $4.3 \cdot 10^5$  recombinant clones and 36 000 recombinant phages from the primary library were plated. Replica filters were differentially screened using radioactively labeled cDNA probes derived from poly-A<sup>+</sup>RNA of the RBT 323/pass.10 (high metastatic) and the RBT 157/pass.1 (low metastatic). Extensive comparison of the resulting autoradiograms (Fig. 1) revealed 22 cDNA clones that seemed to be differentially ex-

pressed; 16 overexpressed in the RBT 323 line and 6 overexpressed in the RBT 157 line. These 22 selected cDNA clones were further evaluated using Northern blots containing 10  $\mu$ g of RBT 157/1, RBT 323/1 and RBT 323/10 RNA. The expression pattern of the most significant clone pV13 is shown in Fig. 2A.

#### 3.2. Expression pattern of pV13

Clone pV13, is clearly overexpressed in RBT 157/1, the line with the lowest metastatic capacity. pV13 detects a single transcript of approx. 1.1 kb (Fig. 2A). The correlation of clone pV13 expression with metastatic capacity was further studied by means of Northern blot analysis of more passages of the rat progression model (Fig. 2B). The passages 1 and 6 of the RBT 157 line, with the moderate metastatic capacity, show both an higher expression level of pV13 compared to the tested passages of the RBT 323 line, with the high metastatic capacity. As a control we

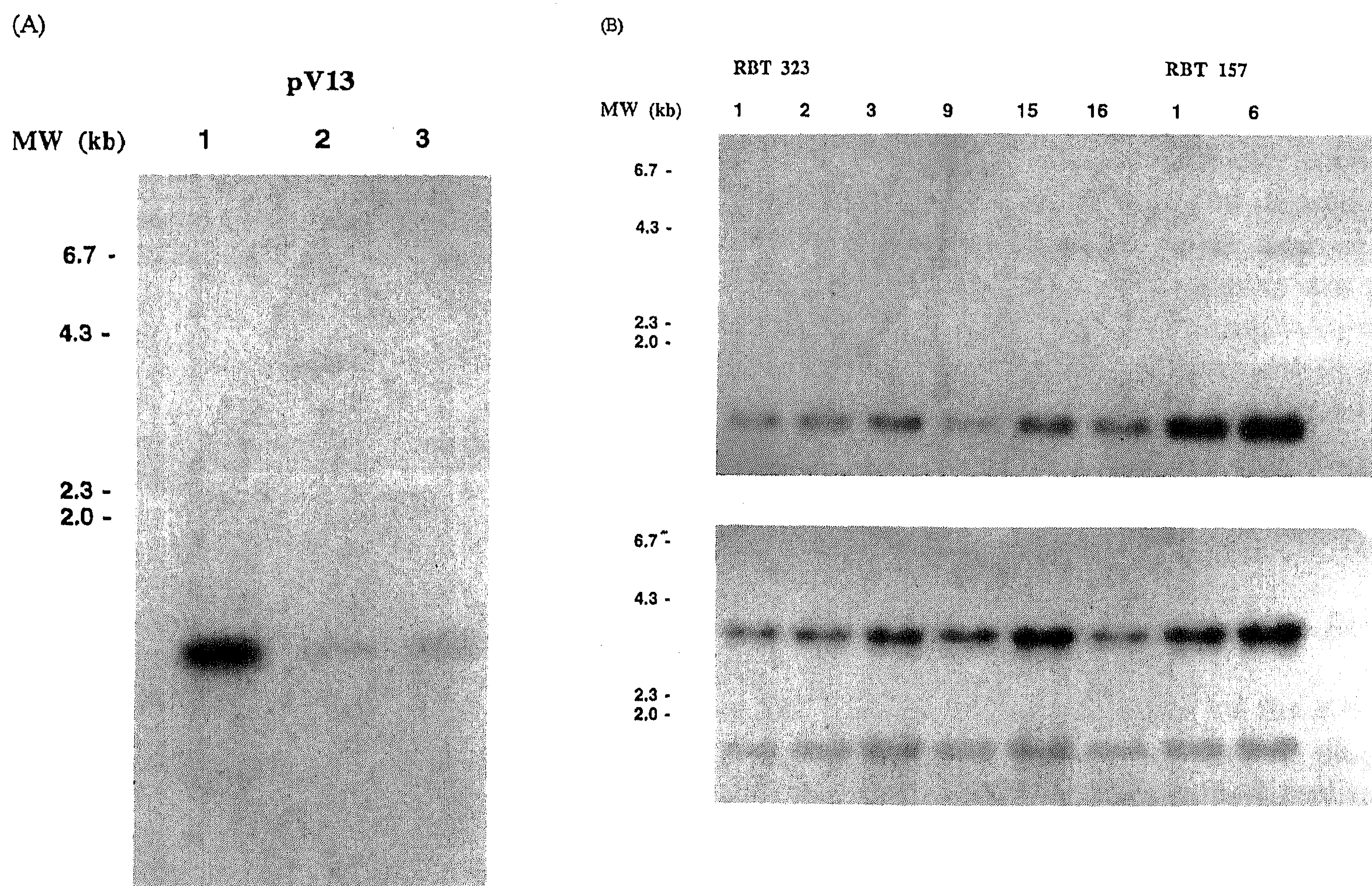


Fig. 2. 10  $\mu$ g of total RNA was glyoxylated, size fractionated on 1% agarose gels and transferred to Hybond-N<sup>+</sup> nylon membranes. A. Secondary screening of the selected cDNA clone pV13 on the passages RBT 157/1 (1), RBT 323/1 (2) and RBT 323/10 (3). Probe pV13 was made of the differentially expressed cDNA clone by random prime labelling reactions. B. Correlation of clone pV13 with metastatic phenotype. The probe was made, using the isolated insert of cDNA clone pV13, by random labelling reaction. rRNA was used as an internal control for the amount of RNA loaded (shown in lower panel (B)).

studied the expression of pV13 in Dunning R-3327 rat prostatic tumors [8] and in normal rat prostate (in normal bladder insufficient RNA was isolated from the tiny layers of urothelium). The higher expression of pV13 in the sublines with lower metastatic capacity, as seen for the bladder tumor model system, was confirmed and in normal rat prostate no expression of pV13 was observed (data not shown).

### 3.3. DNA sequence analysis of pV13

To obtain further information on the cDNA clone, the nucleotide sequences were determined using the Sequenase Version 2.0 sequence system. Computer-assisted comparison of the resulting nucleotide sequences with the EMBL and Gen bank nucleotide sequence databases revealed that pV13 is highly homologous to the Rat Ferritin Heavy chain gene (97%) [9] and the Mouse Ferritin Heavy chain (94%) [10].

## 4. Discussion

In search of new molecular markers with predictive value for the progression of superficial TCC, we compared two different tumor lines of a rat TCC model system at the level of gene expression using differential hybridization analysis. We observed an overexpression of ferritin H in the RBT 157 tumor line which displays the lowest metastatic capacity and the best differentiated phenotype.

Ferritin H combines with the ferritin L subunit to form the iron storage protein ferritin [11]. Ferritin H-rich molecules take up and release iron more readily than L-rich molecules, that are more suited for long-term storage [12]. A relationship between ferritin and cancer has been proposed, since serum ferritin is frequently elevated in patients with cancer [13,14]. Some neoplastic cells also exhibit a remarkably altered ferritin subunit composition (i.e. enrichment for the heavy chain subunit) as compared to their normal counterparts [15,16]. A direct association between oncogenic transformation and ferritin synthesis has been suggested, since the adenovirus E1A oncogene specifically represses ferritin H expression [17]. The observed down regulation of ferritin H expression in the more malignant tumor line RBT 323 is in concordance with this observation. The

correlation between ferritin H expression and a number of differentiation processes shown by others [18,19], is corroborated in this study by the overexpression of ferritin H in the better differentiated tumor line RBT 157. In smooth muscle cells it is proposed that cAMP promotes ferritin H expression resulting in both growth arrest and an enhanced differentiated phenotype [20].

The exact biological consequences of altered ferritin subunit composition are not well understood. It may result in modulation of the iron binding capacity of ferritin and ultimately, the regulatory pools of free iron [21,11]. An enrichment for the ferritin H subunit leads to more efficient iron detoxification and cellular protection against free radical formation [22]. Another mechanism underlying the regulation of ferritin expression has been extensively studied and is exerted posttranscriptionally by specific mRNA-protein interaction between the iron-regulating protein (IRE-BP) and Iron-Responsive Elements (IREs) contained in the 5' untranslated region of ferritin. Iron inactivates the cytoplasmic IRE-BP, thereby preventing its binding to the IRE and consequently derepressing ferritin translation [23].

It should be emphasized that the mechanisms underlying the altered subunit composition of ferritin in neoplastic cells are not elucidated. Ferritin H is located on human chromosome 11 [24], near 11q13 [25], a region that is sometimes amplified in bladder cancer [26]. We have determined that the ferritin H gene is not within the amplicon on 11q13 (data not shown).

Cytogenetic analysis of the RBT lines showed that both of the independently arisen tumors exhibit loss of chromosome 5 [1]. Rat chromosome 5 is syntenic to the major portion of human chromosome 9 (p23-qter) a chromosome that is commonly lost in human TCC. The more aggressive subline RBT 323 has considerably more cytogenetic changes compared to RBT 157. Due to the fact that the comparative genomic map of human and rat is rather restricted [27] it is difficult to speculate about the genes potentially relevant to the observed cytogenetic changes.

Our findings show a correlation of ferritin H overexpression with lower metastatic capacity and better differentiated phenotype in a rat bladder tumor model system. We are currently investigating the value of differential expression of ferritin H as a prognostic

marker in human bladder cancer using quantitative comparative Polymerase Chain Reaction.

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