## Proteome Profiling of Bladder Squamous Cell Carcinomas: Identification of Markers That Define Their Degree of Differentiation


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# Proteome Profiling of Bladder Squamous Cell Carcinomas: Identification of Markers That Define Their Degree of Differentiation[1]

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## ABSTRACT

**One hundred fifty fresh bladder tumors were analyzed blindly by two-dimensional PAGE in combination with proteome identification techniques (microsequencing and mass spectrometry) and immunofluorescence of cryostat sections. Of these, six showed protein expression patterns corresponding to squamous cell carcinomas (SCCs). All tumors were already invasive at the time of presentation, and in most cases, the histopathological grade could not be determined with certainty. The more differentiated of the tumors included SCC 589-1, a lesion showing extensive keratinization, and 536-1, a pure SCC that resembled normal skin growing invasively into the muscle. Both tumors expressed keratins 5, 6, 10, 14, 16, 17, and 20, as well as the differentiation-associated proteins psoriasin, psoriasis-associated fatty acid-binding protein (PA-FABP), and galectin 7. SCC 589-1, however, exhibited higher levels of keratin 10, PA-FABP, and galectin 7 and, in addition, expressed keratins 13, 15, and 19, which were not detected in the pure SCC. Involucrin, glutathione *S*-transferase $\pi$, stratifin (14-3-3 $\sigma$), and the SCC antigen 1, on the other hand, were less abundant in SCC 589-1. In comparison, less-differentiated tumors did not express keratin 10 and were characterized by a decreased expression of keratin 14, psoriasin, PA-FABP, galectin 7, and stratifin (14-3-3 $\sigma$). Indeed, two of these lesions (553-1 and 651-1) could be readily lined up in order of decreasing degree of differentiation based on the expression of these markers. The degree of differentiation of the other two SCCs could not be assessed with certainty because they may represent special cases (SCC 646-1, solid tumor; SCC 485-1, special differentiation pattern). All six SCCs externalized psoriasin to the urine, supporting the contention that this protein, alone or in combination with other polypeptides, may represent a useful marker for the early detection of these lesions.**

## INTRODUCTION

Bladder cancer comprises a broad spectrum of tumors that includes TCCs,[3] SCCs, adenocarcinomas, small cell carcinomas, and leiomyomas (1, 2). TCCs are by far the more prevalent tumors, and they represent nearly 90% of all bladder cancers in the Western Hemisphere (3). SCCs on the other hand, encompass a small percentage (2–3%) of all bladder tumors diagnosed in Europe and America (4), but are very frequent (80%) in areas of Africa and the Middle East where *Schistosoma hematobium*, a parasite that induces bladder SCCs in humans, is prevalent (5). The histogenesis of SCCs is unclear, although these lesions may arise from extensive squamous differentiation of TCCs (*i.e.*, carcinoma *in situ* or high-grade papillary TCCs) or from neoplastic transformation (on the basis of squamous metaplasia) of the bladder urothelium (6). SCCs are highly malignant, and the success of treatment relies heavily on early detection.

SCCs are composed of one cell type that resembles keratinocytes (7–9), and they exhibit distinct squamous characteristics, such as "pearl" formation and keratohyalin bodies (7, 8). Grading of these tumors is subjective and takes into consideration the degree of nuclear polymorphism, nuclear:cytoplasmic ratio, chromatin clumping, and number of mitotic cells (1). These parameters, however, are difficult to evaluate with precision, and as a result, it is often not possible to distinguish between poorly differentiated TCCs with areas of squamous differentiation and highly undifferentiated SCCs (1). In addition, different areas from the same tumor often show variable degrees of differentiation.

In an effort to achieve a more reliable and objective classification of bladder SCCs and eventually shed some light on the molecular mechanisms underlying progression, we have considered the possibility of using protein expression profiles of these lesions as fingerprints to identify tumor subtypes and to define their grade of atypia. Considering the striking similarity between SCC cells and keratinocytes (9), these studies are expected to be facilitated by extensive protein data already available in human two-dimensional PAGE databases of keratinocyte and urothelial cell proteins (10, 11). Eventually, as a result of this research, it may be possible to predict tumor course and to identify reliable criteria for planning of treatment.

Optimally, such a study should be applied to the analysis of fresh tumors rather than to cultured cells prepared from these lesions because the latter may undergo changes in their expression patterns as a result of *in vitro* cultivation. A study of fresh SCC tumors, on the other hand, requires markers to facilitate the interpretation of patterns resulting from heterogeneous samples and the blind analysis of many samples, because these tumors are rare. Here, we present a proteome profiling study of 6 SCCs identified blindly among 150 tumors analyzed by two-dimensional PAGE during a period of 1 year. In addition to revealing protein markers that shed some light on the degree of differentiation and origin of these tumors, the results support the contention that the chemoattractant protein psoriasin (12–14), which is externalized to the urine by these tumors (9), may represent a promising marker for the early detection of these lesions.

## MATERIALS AND METHODS

**Tumor Biopsies and Processing.** Fresh tumors (Skejby Hospital) were placed immediately on ice and transported to the laboratory. Tumor pieces for cryostat sections were kept at −80°C.

**Preparation of Tumor Samples for Electrophoresis.** Tumors cleaned of clots and contaminating tissue were minced in small pieces with the aid of a scalpel and homogenized (glass homogenizer) in lysis solution (15) prior to electrophoresis. In a few cases, the tumor pieces were labeled with [$^{35}$S]methionine for 14–16 h in a 10-ml sterile plastic conical tube containing 0.2 ml of modified Eagle's medium lacking methionine and containing 2% dialyzed (against 0.95% NaCl) FCS and 100 $\mu$Ci of [$^{35}$S]methionine (SJ204, Amersham). At the end of the labeling period, the pieces were centrifuged at 2000 × *g* for 2 min, resuspended in 0.3–0.4 ml of lysis solution, and

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[3] The abbreviations used are: TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; PA-FABP, psoriasis-associated fatty acid-binding protein; SCCA1, squamous cell carcinoma antigen 1; mAb, monoclonal antibody.

homogenized in a small glass homogenizer. Samples were stored at −20°C until use. Normal bladder urothelium was scraped with the aid of an scalpel and labeled with [$^{35}$S]methionine as described above (9).

**Urine Collection and Processing.** Voided urine collected the day before the operation was processed for gel electrophoresis as described by Rasmussen *et al.* (16).

**Abnormally Differentiated Keratinocytes.** Strips of normal skin were washed three times in HBSS and placed in 0.25% trypsin in HBSS (Life Technologies, Inc., Paisley, Scotland; 1:250) at 4°C for at least 24 h. Following incubation, the strips were washed three times in serum-free keratinocyte medium (Life Technologies, Inc.) supplemented with epidermal growth factor (5 ng/ml, Life Technologies, Inc.) and bovine pituitary extract (35–50 μg/ml). The epidermis was then detached from the dermis and resuspended in 8 ml of the same medium. Following vigorous shaking, the cells were plated in 35-mm culture dishes coated with a dermal extract (17). Keratinocytes were fed with fresh medium every day, and loosely attached cells were eliminated by pipetting the medium up and down with the aid of an automatic pipette. Primary keratinocytes were incubated in DMEM containing 10% FCS on the fifth day after plating. Keratinocytes were incubated for 10 days in this medium prior to [$^{35}$S]methionine labeling.

**Two-Dimensional Gel Electrophoresis and Immunoblotting.** Two-dimensional PAGE was performed according to Celis *et al.* (11, 18). Two-dimensional gel immunoblotting was performed according to published procedures (19).

**Protein Identification.** Proteins were identified using a combination of procedures that included microsequencing and mass spectrometry (20), two-dimensional gel Western immunoblotting, and enhanced chemiluminescence detection (19, 21), as well as by comparison with the master two-dimensional gel image of human keratinocyte proteins (10, 11).

**Preparation of mAbs against Psoriasin.** mAbs against recombinant human psoriasin (13) were prepared essentially as described by Celis *et al.* (22). A BALB/c mouse was immunized with recombinant psoriasin prepared in *Escherichia coli*, and the hybridoma supernatants were screened by dot blotting using the recombinant psoriasin. A positive hybridoma was cloned three times by limited dilution, and the specificity of the antibody was determined by two-dimensional gel immunoblotting of whole cell extracts prepared from human keratinocytes.

**Other Antibodies.** mAbs against keratins 10 and 19 were purchased from Cappel. mAb BG3C8, which reacts specifically with epidermal basal cells (23), was a kind gift from A. Celis. (Department of Medical Biochemistry, University of Aarhus, Denmerk).

**Indirect Immunofluorescence.** Tumor biopsies frozen in isopentane were sectioned on a Reichert-Jung cryostat. Eight-μm sections placed on coverslips were washed three times with HBSS and treated for 5 min at −20°C with methanol (9). After being washed extensively with HBSS, the coverslips were covered with 20 μl of the primary antibody and incubated for 60 min at 37°C in a humid environment. The coverslips were washed several times with HBSS and covered with 20 μl of Rhodamine-conjugated secondary antibody (diluted 1:50 in HBSS). After a 60-min incubation at 37°C in a humid environment, the coverslips were washed thoroughly with HBSS and mounted in Gelvatol (Air Products, Utrecht, the Netherlands). Observations were made on a Zeiss photomicroscope equipped with epifluorescence and phase-contrast optics.

## RESULTS

**Proteome Profiling of SCCs.** One hundred fifty fresh bladder tumors removed at Skejby Hospital during 1996 and the beginning of 1997 were analyzed blindly by two-dimensional PAGE as described in "Materials and Methods." Of these, six showed protein expression patterns corresponding to SCCs (Ref. 9; Table 1), a fact that was later confirmed by the pathologist's report. All tumors were already invasive at the time of presentation, and in most cases, the histopathological grade could not be determined with certainty. In addition to the six SCCs, we detected one tumor that expressed a keratinocyte-like protein pattern and that was classified as a urothelial grade III lesion, mostly differentiated into SCC.

**SCC 589-1.** SCC 589-1, a lesion with extensive keratinization, showed the highest degree of differentiation as judged by the high expression of keratin 10 (Fig. 1*A*; Fig. 2*A*; Table 1), a protein that is expressed only by cells in the upper layers of the epidermis (24). The presence of this keratin was further confirmed by indirect immunofluorescence staining of cryostat sections, which revealed positive cells in the most differentiated areas of the tumor, in particular the pearls (Fig. 3*A*). As shown in Fig. 1*A*, the protein fingerprint of SCC 589-1 is very similar to that of abnormally differentiated human keratinocytes (Fig. 1*B*; Ref. 25) but differs significantly from those of noncultured normal urothelium (Fig. 1*C*) or low-grade papillary TCCs (Fig. 1*D*; Ref. 26). Like abnormally differentiated keratinocytes, SCC 589-1 expressed keratins 5, 6, 14, 15, 16, 17, and 19 (see also Fig. 3*B*: not all cells are stained), as well as high levels of the differentiation-associated proteins psoriasin (S100A7; Refs. 12–14), a partially externalized calcium-binding (27) and chemotactic protein (14) localized mainly to the pearls (Fig. 3*C*), PA-FABP (17), galectin 7 (Ref. 28; Fig. 2*A*; this protein does not contain methionine), and the calgranulins (migration inhibitory factor-related proteins 8 and 14). The identity of these proteins was determined by immunoblotting and in some cases by microsequencing, mass spectrometry, and comparison with the master image of human keratinocyte proteins currently available on the World Wide Web (10, 11). In contrast to differentiated keratinocytes, SCC 589-1 expressed keratin 13, which was observed in the more differentiated areas of the tumor as judged by indirect immunofluorescence (results not shown), and keratin 20 and exhibited lower levels of involucrin and of SCCA1 (Ref. 29; Fig. 1, *A* and *B*).

**SCC 536-1.** Tumor 536-1 was recorded as a highly differentiated pure SCC that resembled normal skin growing invasively into the muscle. This tumor shared the highest similarity with epidermal keratinocytes both in terms of protein expression and morphology, in particular the epidermis-like differentiation of the mucosa contiguous to the tumor. SCC 536-1 expressed abundant levels of keratins 5, 6, 14, 16, 17, and 20; psoriasin; and the calgranulins as determined by two-dimensional PAGE analysis, but it exhibited lower levels of keratin 10, PA-FABP, and galectin 7 as compared to SCC 589-1

Table 1 *Expression of keratins and differentiation markers by SCCs*[a]

| SCC | Keratin 10 | Keratin 13 | Keratin 14 | Keratin 19 | Psoriasin (S100A7) | PA-FABP | Galectin 7 | Stratifin (14-3-3 σ) | Comments from the pathologist's report |
|---|---|---|---|---|---|---|---|---|---|
| 589-1 | ++ | + | ++ | + | ++ | ++ | ++ | ++ | SCC with keratinization |
| 536-1 | ± | − | ++ | − | ++ | + | + | +++ | Pure SCC with epidermal metaplasia, keratinizing |
| 553-1 | − | ++ | + | ++ | ± | + | ± | + | SCC with keratinization |
| 651-1 | − | − | ± | ++ | ±[b] | ± | − | ± | SCC with keratinization |
| 646-1 | ND | − | + | ± | ±[c] | ± | − | + | SCC, solid (large variation in nuclear size) |
| 485-1 | − | − | ± | − | ± | + | ± | + | SCC without keratinization, special differentiation pattern |

[a] The levels are estimated based on visual analysis of Coomassie Brilliant Blue-stained two-dimensional gels and are relative to SCC 589-1. +++, highest; ++, very high; +, high; ±, low; −, below level of detection; ND, not determined (mainly due the low quality of the two-dimensional gel in that particular area). In several cases, the data were confirmed by similar analysis of autoradiograms.

[b] ±, but lower than SCC 553-1.

[c] ±, but lower than SCC 651-1.

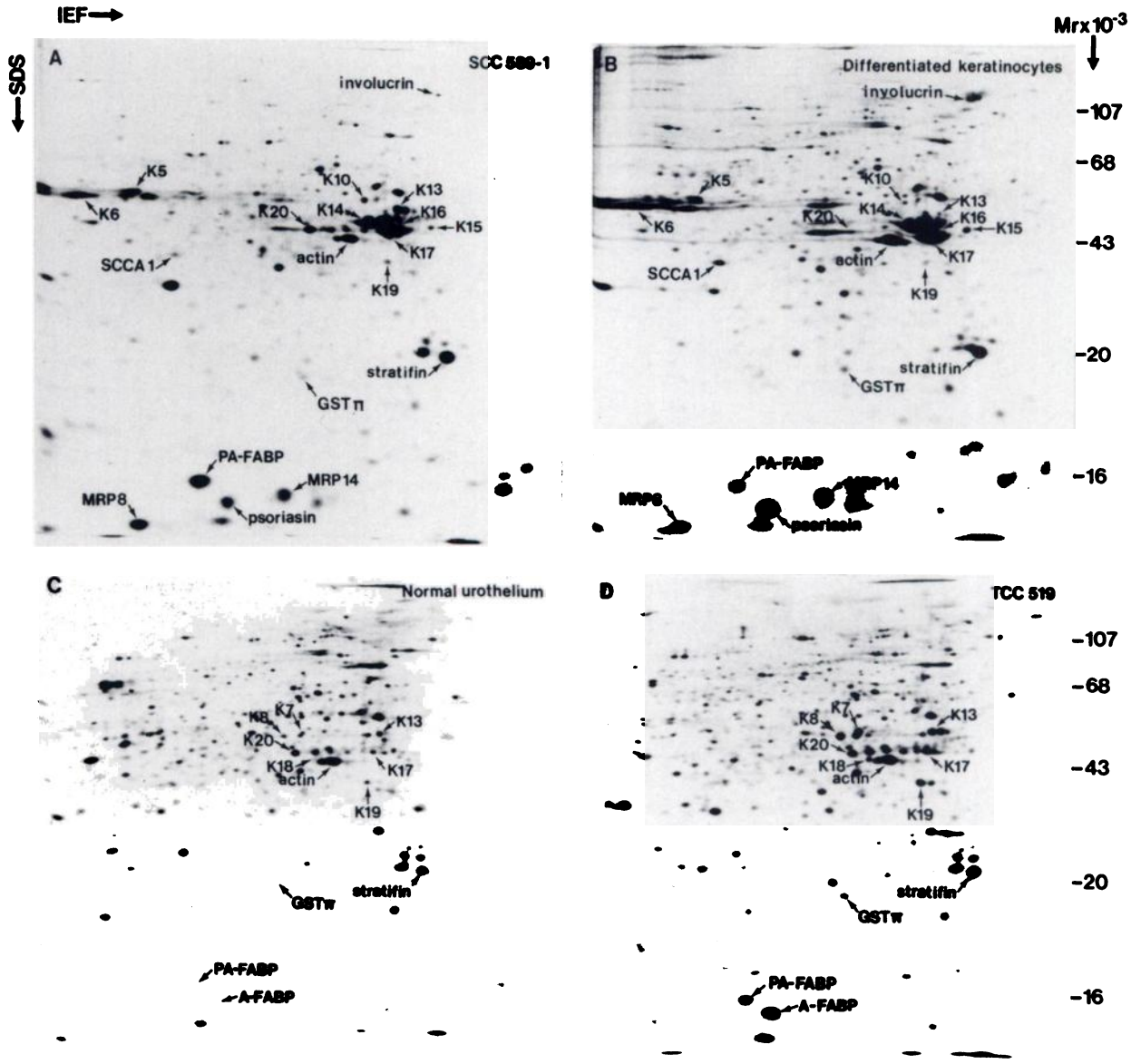


Fig. 1. Isoelectric focusing two-dimensional gels of [$^{35}$S]methionine-labeled proteins. *A*, SCC 589-1; *B*, abnormally differentiated keratinocytes (see "Materials and Methods"); *C*, normal urothelium prepared as described in Celis *et al.* (9); *D*, TCC 519 (papillary, grade I). Fresh tumors, normal urothelium, and differentiated keratinocytes were labeled for 14–16 h with [$^{35}$S]methionine as described in "Materials and Methods."

(Fig. 2, *A* and *B*; Table 1). SCC 536-1 did not express detectable levels of keratins 13, 15, and 19 (see also Fig. 3*D*) but exhibited higher levels of involucrin, stratifin (14-3-3 $\sigma$; Ref. 30), glutathione *S*-transferase $\pi$, and SCCA1 relative to SCC 589-1 (compare Fig. 2*A* and Fig. 2*B*). As judged by immunofluorescence, psoriasin was observed both in the more differentiated areas of the keratinizing epidermal structures (Fig. 3*E*) and in the pearls (results not shown; see Fig. 3*C*).

**Less-Differentiated SCCs.** None of the less-differentiated tumors (SCCs 553-1, 651-1, 646-1, and 485-1; Table 1) expressed keratin 10, and all exhibited reduced levels of keratins, 5, 6, and 14; psoriasin; PA-FABP (with the exception of 485-1); galectin 7; and stratifin (14-3-3 $\sigma$) as compared to their more-differentiated counterparts (SCC 589-1 and 536-1). Two of these lesions (553-1 and 651-1) could be readily lined up in order of decreasing degree of differentiation based mainly on the expression of keratin 14, psoriasin, PA-FABP, galectin 7, and stratifin (Fig. 2, *C* and *D*; Table 1). Both of these lesions expressed higher levels of keratin 19 than did the more differentiated SCC 589-1, but SCC 651-1 did not express keratins 13 and 15 (Fig. 2, *C* and *D*; Table 1). The degree of differentiation of the other two SCCs could not be assessed with certainty because they may represent special cases. Tumor 646-1 was reported as being a solid carcinoma with large variation in nuclear size. The tumor showed reduced levels of keratin 19 as compared to SCCs 553-1 and 651-1, and like the latter, it did not express keratin 13 (results not shown). SCC 485-1, on the other hand, was recorded as having a special pattern of differentiation with no keratinization. This lesion did not express keratin 19 or 13 and exhibited reduced levels of keratin 14 (Fig. 2*E*; Table 1). It should be mentioned that none of the tumors mentioned above expressed keratin 7 or 18 as juged by two-dimensional PAGE analysis and immunofluorescence (results not shown).

**Urothelial Tumor with Squamous Metaplasia.** Tumor 693-1 corresponded to a urothelial grade III lesion with extensive squamous differentiation. Two-dimensional PAGE analysis of this lesion re-

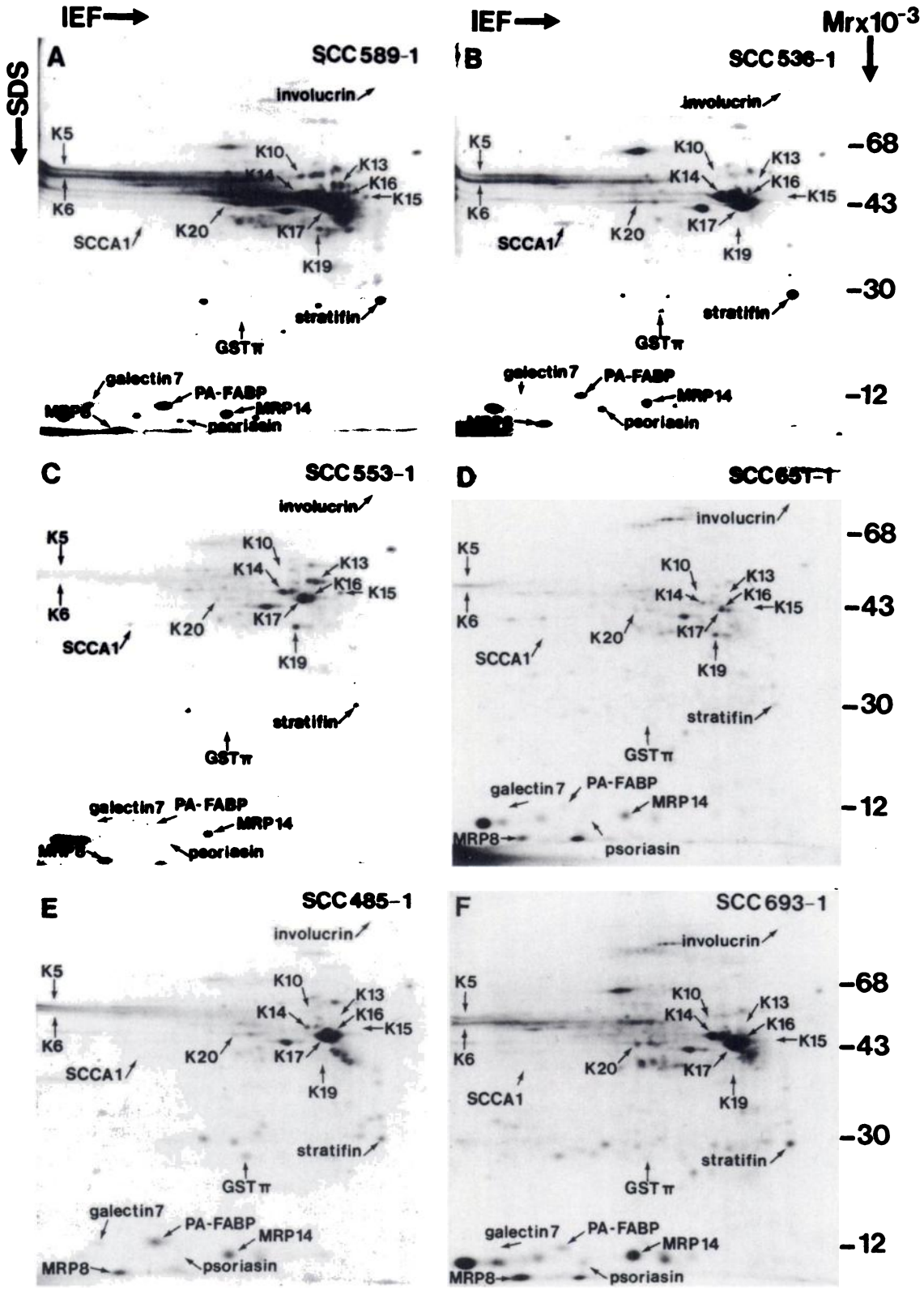


Fig. 2. Isoelectric focusing two-dimensional gels of Coomassie Brilliant Blue-stained proteins. *A*, SCC 589-1; *B*, SCC 536-1; *C*, SCC 553-1; *D*, SCC 651-1; *E*, 485-1; *F*, 693-1. Fresh tumors were homogenized in lysis solution as described in "Materials and Methods."

vealed abundant expression of keratins 14 and 16, as well as of the differentiation-associated proteins psoriasin and PA-FABP (Fig. 2*F*). The tumor as a whole showed very small amounts of keratin 19 as judged by two-dimensional PAGE analysis (Fig. 2*F*); this is most likely due to the presence of a small area of the lesion that stained positively with the keratin 19 antibody (Fig. 3*F*). In contrast to normal urothelium (26), this area did not stain with antibodies against keratins 7 and 18 (not shown), suggesting that major changes in protein

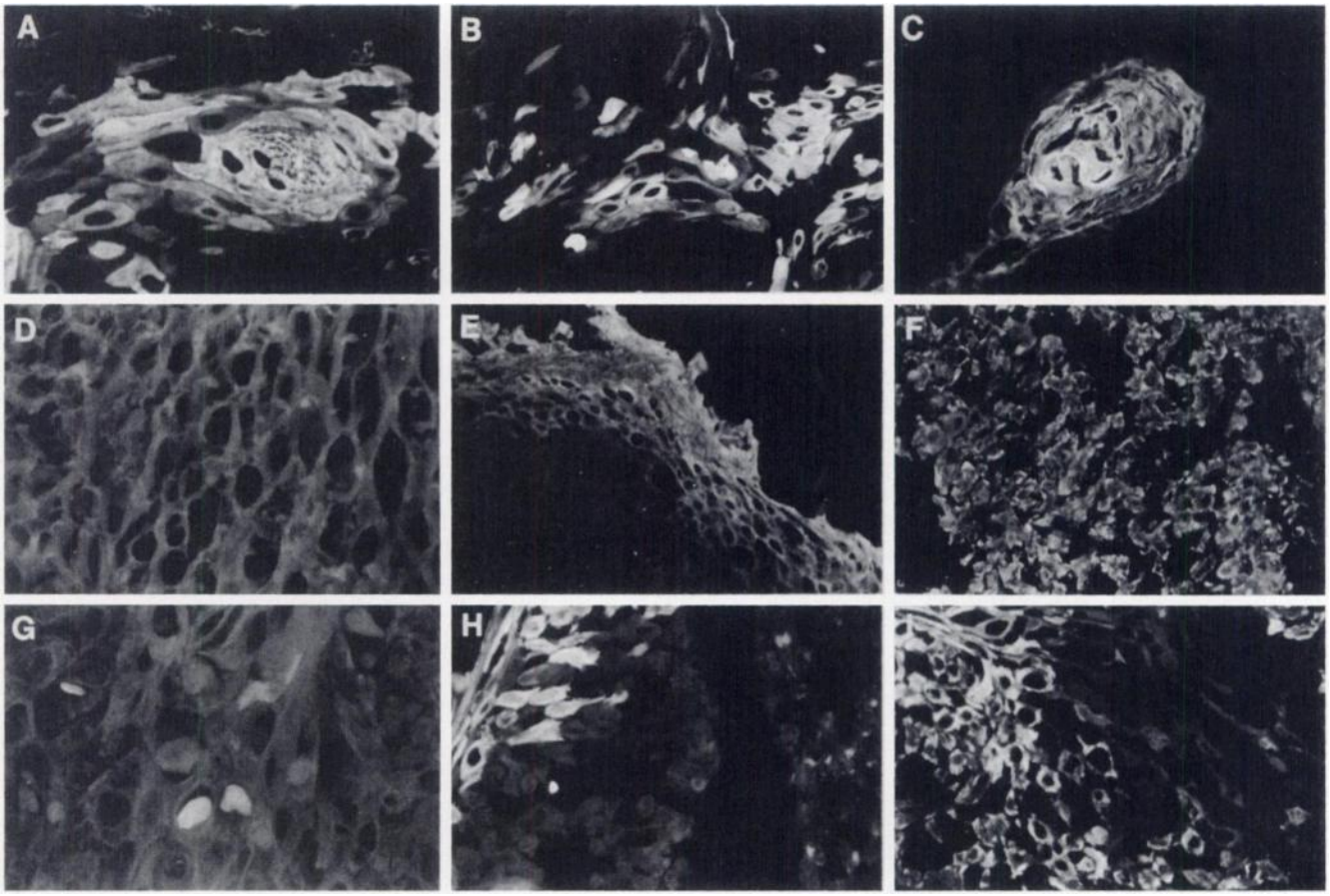


Fig. 3. Indirect immunofluorescence staining of formaldehyde-fixed cryostat sections. *A–C*, SCC 589-1 reacted with antibodies against keratin 10 (*A*), keratin 19 (*B*), and psoriasin (*C*); *D* and *E*, SCC 536-1 reacted with antibodies against keratin 19 and psoriasin, respectively; *F* and *G*, different areas of SCC 693-1 reacted with antibodies against keratin 19; *H* and *I*, SCC 553-1 reacted with antibodies against keratin 19 and mAb BG3C8 (basal cell specific), respectively.

expression have taken place. As shown in Fig. 3*G*, the main area of the tumor did not stain with the keratin 19 antibody in line with the two-dimensional PAGE analysis.

**SCCs Externalize Psoriasin to the Urine.** Previously, we reported the externalization of psoriasin to the urine of patients bearing SCCs (9). To verify this observation, which was based on a limited study, we screened the urine of the six SCC patients by two-dimensional gel immunoblotting using mAbs and polyclonal antibodies raised against psoriasin. A positive reaction was observed in all cases, supporting the contention that this protein, alone or in combination with other polypeptides, may represent a useful marker for the early detection of these lesions. Examples of two-dimensional gel immunoblots of the urine from patients bearing the less-differentiated lesions (SCC 485-1 and 651-1) are shown in Fig. 4, *A* and *C*, respectively. For comparison, a negative control (TCC 563-1) is shown in Fig. 4*E*. Immunoblots of the corresponding tumors are shown in Fig. 4, *B*, *D*, and *F*.

## DISCUSSION

The lack of histological criteria for reproducible and objective grading of SCCs, as well as of markers for early detection, has presented major obstacles in the treatment of these lesions (6). In this report, we have attempted to address these issues by combining a proteome profiling study of fresh tumors with an immunofluorescence analysis of cryostat sections to reveal subtle changes in protein distribution that are not obvious from the two-dimensional PAGE protein analysis. In general, our studies have shown that it is possible to differentiate SCCs based on their protein profiles, and in addition, they have underlined the value of proteins such as keratins 10 and 14, psoriasin, PA-FABP, galectin 7, and stratifin (14-3-3 $\sigma$) as markers to assess their degree of differentiation. Previous studies in our labora-

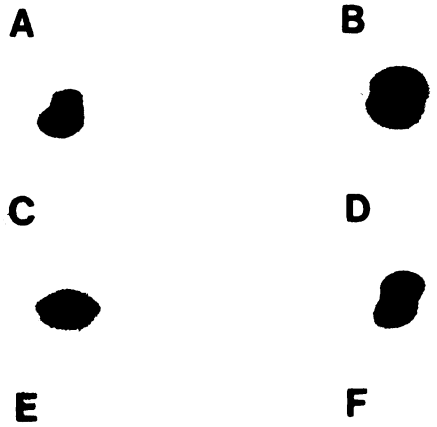


Fig. 4. Two-dimensional gel Western blots reacted with antibodies against psoriasin. *A*, urine 485-1; *B*, SCC 485-1; *C*, urine 651-1; *D*, SCC 651-1; *E*, urine 563-1; *F*, TCC 563-1. The equivalent of 1 ml of urine was applied to the the first-dimension gels.

tory have shown that the levels of these proteins are highly sensitive to differentiation and cell transformation (25, 31). Likewise, there is evidence indicating that keratin 14 expression is down-regulated in an HPV-16-immortalized cervical SCC cell line following tumorigenic transformation with v-Ha-ras DNA (32), as well as in premalignant and malignant oral SCC tissue (33). Reduced synthesis of galectins 1 and 3 has been reported in head and neck SCCs, suggesting that the expression of the galectin family of proteins is associated with differentiation (34).

The histogenesis of SCCs is at present unclear, although these lesions are thought to arise from extensive squamous differentiation of TCCs (*i.e.*, carcinoma *in situ* or high-grade papillary TCCs) and from neoplastic transformation (on the basis of squamous metaplasia) of the bladder urothelium (pure SCCs; Ref. 6). One of the tumors analyzed in this study corresponded to a pure SCC (SCC 536-1), and therefore, its protein profile may serve as a reference to speculate on the putative origin of the other tumors. The most striking differences between the pure SCC and the well-differentiated SCC 589-1 was the lack of expression of keratins 13 and 19 by the former. Because these keratins are not expressed by epidermal keratinocytes and are abundant in transitional epithelium, we favor the idea that SCC 589-1 arose as a result of extensive squamous differentiation of a preexisting TCC. The latter may also be true for tumors 553-1 and 651-1 because both expressed high levels of keratin 19, even though only SCC 553-1 exhibited keratin 13, a protein that has been associated with differentiation in head and neck SCCs (35). Indeed, immunofluorescence analysis of SCC 553-1 showed that the mucosa contiguous to the tumor stained abnormally with antibodies against keratin 19 (Fig. 3*H*; only some cells stained), 7 (no staining; not shown), 18 (no staining; not shown), and a stratified epithelia basal cell antigen (mAb BG3C8, Fig. 3*I*; staining of some cells) that we have previously shown reacts with all cells in SCCs (9). These results contrast with those observed in normal urothelium, which shows positive staining of all cells with keratins 19, 7, and 18 antibodies, but is negative for the basal cell marker (results not shown; Ref. 9).

Interestingly, the analysis of tumor 693-1, which was reported as a urothelial grade III (papillary) lesion with extensive squamous metaplasia, revealed a protein expression profile that was strikingly similar to that of the well-differentiated pure SCC. The bulk of this tumor did not express keratin 19 or 13 but showed decreased expression of involucrin, SCCA1, and glutathione *S*-transferase $\pi$ as compared to SCC 536-1. Immunofluorescence analysis of cryostat sections from this tumor revealed a marginal area of the lesion that stained positively with keratin 19 and the basal cell antibody. These cells, however, did not stain with antibodies against keratins 7 and 18, indicating that major changes in protein expression have taken place. Clearly, SCCs derived from extensive differentiation of highly undifferentiated papillary TCCs pose a challenging problem, and further studies are needed before one can attempt meaningful comparisons with other SCC types. Work is currently under way to produce a battery of mAbs against some of the markers described in this study in an effort to facilitate, in retrospective studies, the analysis of various SCCs.

An important goal of our studies has been to identify tumor-specific proteins that are externalized to the urine and that may serve as useful markers for the early detection of SCCs. Here, we have presented further evidence pointing toward psoriasin as such a potential biomarker, alone or in combination with other proteins (9). We foresee, however, problems with reversible benign metaplastic lesions, which may temporarily express this protein. Indeed, we have previously shown that the frequent presence of stratified epithelia in the female trigone (36), which has been attributed to estrogen stimulation, may lead to false positives (9). Immunoblotting experiments using psoriasin antibodies have indicated that this protein, when present in the urine, may only be derived from squamous metaplasia and/or SCCs.[4]

Considering that bladder cancer is a field disease, we (9) and others (37) believe that a great deal of important information may be derived from the analysis of random biopsies. Accordingly, we have started a systematic two-dimensional PAGE analysis of fresh random biopsies labeled with [$^{35}$S]methionine (cellular and externalized proteins) in an effort to define a "normal" baseline that may be useful in pinpointing premalignant lesions. The latter, in combination with information stored in two-dimensional PAGE databases (11), may form the basis for identifying intermediate biomarkers for the early and reliable detection of bladder tumors, as well as for revealing signaling pathways and components that are affected at various stages of tumor progression. These biomarkers are expected not only to be important for drug discovery, but also to provide a better platform for developing treatment and prevention strategies. Thus far, the two-dimensional gel analysis of radioactively labeled random biopsies indicates that the approach is sound, although a meaningful outcome of these studies can only be expected following the analysis of many hundreds of fresh specimens.[5]

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