CANCER GENOMICS & PROTEOMICS *1*: 455-464 (2004)

# Effects of Tumor Necrosis Factor-α (TNFα) and Interferon-γ (IFNγ) on Gene Expression Profiles in Bladder Carcinoma Cells Using Oligonucleotide Microarray Analysis

PIERRE CHAMPELOVIER[1], MICHÈLE EL ATIFI[2], FREDERIC MANTEL[2], SANDRINE MICHALLAT[2], ANNICK SIMON[3], BÉATRICE ROSTAING[2], FRANCOIS BERGER[2] and DANIEL SEIGNEURIN[1]

*[1]Laboratoire de Cytologie, Département d'Anatomie et de Cytologie Pathologique and*
*[3]Laboratoire d'Hématologie, Département de Biologie et de Pathologie de la Cellule,*
*Centre Hospitalier Universitaire de Grenoble, Hôpital Albert Michallon, BP 217, 38043, Grenoble cedex;*
*[2]Equipe Transcriptome, INSERM U318, Laboratoire de Neurosciences Précliniques,*
*Université Joseph Fourier, 38000, Grenoble, France*

**Abstract.** *Background: TNFα and IFNγ, two main cytokines secreted in the urine of bladder cancer patients after Bacillus Calmette Guerin immunotherapy (BCG therapy), exert various responses ranging from growth arrest, apoptosis, phenotypic changes and differentiation. Materials and Methods: To identify their transcriptional and translational targets, the highly sensitive bladder cancer cell line (RT112) was treated for 24 hours with increasing doses of IFNγ or TNFα and analyzed for cellular and molecular changes using a cDNA microarray technique (Transcriptome) containing 800 genes. Results: High doses (>10 ng/ml) induced an apoptotic cell death, whereas low doses (<5 ng/ml) induced a survival program. TNFα-inducible genes, IFNγ-inducible genes and genes modulated by TNFα and IFNγ together were identified. All were related to the tumor progression program including cell proliferation, apoptosis/survival, angiogenesis and metastatic processes. Conclusion: These results suggest that the transcriptomic approach could be a good methodology to determine the molecular mechanisms involved in bladder tumor progression processes in relation to a low response to BCG treatment. However, mRNA and protein expression did not always correlate, suggesting that translational regulation is a vital process in bladder tumor progression.*

Transitional cell carcinoma (TCC) of the bladder is the fifth most common cancer in humans. TCC has been classified into two groups including superficial (pTa) and invasive (pT1 to pT4) with distinct clinical behaviors. The standard initial therapy for superficial pTa bladder cancers is transurethral resection with or without adjuvant Bacillus Calmette Guerin (BCG) therapy, whereas invasive pT1 to pT4 bladder cancers are treated with transurethral resection with adjuvant BCG therapy or chemotherapy. Since the first study reported by Morales *et al.* (1), all the published reports have confirmed the efficacy of intravesical BCG therapy in reducing both the progression of superficial bladder cancer and the recurrence of invasive tumors (2). The mechanisms by which BCG mediates antitumor activity are not all understood. However, recent studies showed that TCC patients that respond to BCG therapy have a cellular and humoral response towards tumor cells. The basis of this immune response is associated with the secretion of several cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, M-CSF/CSF1, TNFα and IFNγ) (3–6). Following BCG therapy, the expression of the major histocompatibility class II and ICAM-1/CD54 antigens on urothelial cancer cells increased, suggesting that IFNγ induced action. Moreover, the antitumor activity and the cytotoxicity of IFNγ against bladder tumor cells have been investigated *in vitro* and clinical studies have shown the therapeutic efficacy of IFNγ against recurrence of TCC (7-9). However, *in vivo*, IFNγ may enhance the metastatic potential of cancer cells and, *in vitro,* low doses of IFNγ induce an epithelial to mesenchymal (EMT) process and tumor progression (10, 11). IFNγ also induces TNFα secretion and increases the expression of TNF receptors, which could explain the synergistic effect of the two cytokines (12, 13). TNFα was originally characterized as an antitumor agent and a cytotoxic factor for many malignant cells (14). However, expression of endogenous TNFα may act as a protective protein against the cytotoxicity of exogenous TNFα, possibly through NF-kB inhibition (15, 16).

The multiple molecular events that occur when bladder cancer patients are treated using BCG therapy have been

*Correspondence to:* Dr Pierre Champelovier, Laboratoire de Cytologie, Centre Hospitalier Universitaire de Grenoble, BP 217X-38043, Grenoble cedex 09, France. Tel: 33 4 76 76 54 89, Fax: 33 4 76 76 59 92, e-mail: PChampelovier@chu-grenoble.fr

*Key Words:* TNFα, IFNγ, survival, microarray.

examined either at the protein level (cytokines, growth factors, chemokines) or at the single gene level (17). Today, new technologies have been developed to analyze mRNA expression of a large number of genes, with the cDNA microarray techniques making it possible to simultaneously compare the expression of thousands of genes with a single hybridization. Due to their sensitivity (a single mRNA copy per cell), these technologies can detect the regulatory function of various molecules. Recent investigations showed that these techniques can be used to identify the gene expression pattern, providing the new biological insight required for the development of a new therapy for bladder cancer (18–20). Moreover, cDNA microarrays have been used to study the differential expression of mRNAs after activation either with cytokines and growth factors or with chemical molecules used in chemotherapy (21–24).

Because some bladder cancer patients treated with BCG therapy will develop muscle invasion, metastasis and resistance to this treatment, it is crucial to investigate the effects of cytokines at the transcriptional level to identify their target genes in relation to the tumor progression process. As TNFα and IFNγ are the main mediators of humoral response, we used a DNA microarray containing the probes for 800 human genes to determine changes in mRNA expression after *in vitro* treatment of bladder cancer cells that are able to progress. Our results suggest that bladder cancer patients with a slight response to immunotherapy (low levels of TNFα or IFNγ in the urine) could progress and tumors could metastasize as a result of the effects of these low doses of TNFα or IFNγ on these processes.

## Materials and Methods

*Cell cultures.* The RT112 cell line was derived from a pT1G2 bladder tumor and was maintained in RPMI 1640 medium with 10% inactivated fetal calf serum (FCS) (Gibco BRL, Eragny, France), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. In the induced cultures, RT112 cells were grown in RPMI 1640 medium with 5% FCS. TNFα or INFγ were added to the subconfluent proliferating culture of the RT112 cells for 24 hours. Cells were treated with trypsin, washed twice in PBS and used either for biological assays or for mRNA analysis.

*Drugs and reagents.* Recombinant human (rh) TNFα and INFγ, (R&D Systems, Minneapolis, MN, USA) were dissolved as specified by the manufacturer and diluted to appropriate final concentrations in RPMI 1640 medium.

*Apoptosis determination.* Increasing doses of TNFα or INFγ were added to the subconfluent proliferating culture of the RT112 cells for 24-48 hours. Nonadherent and trypsin-treated cells were mixed, washed twice in PBS and analyzed using two techniques, as previously described (11).

*Oligonucleotide microarray analysis (transcriptional activity). Array features:* The microarray analyses were performed using long oligonucleotide nylon filters with 500-μm spot spacing. The 70-mer oligonucleotides were obtained from Operon Company GmbH (Cologne, Germany). The DNA probes were printed in duplicate onto the membrane and probes for the standard RNA (*Oryza sativa* gene) were printed in 48 locations onto the filter. An anti-sense DNA probe for the RNA standard was used as negative signal to control the hybridization specificity. The quality of the filters and the quantities of printed DNA were determined using the polynucleotide kinase labelling method of El Atifi *et al.* (25).

*Samples, hybridization and expression measurements:* Total RNAs were extracted from cells using the RNAgents® Total RNA Isolation System (Promega, Madison, WI, USA). The quantity and the quality of the extracted RNAs were checked using RNA LabChip and BioAnalyser 2100 (Agilent Technologies, Palo Alto, CA, USA). RNA samples were stored at –80˚C until analysis. For transcriptomic analysis, 3 μg of total RNA sample and 0.2 ng of standard mRNA sample were reverse transcribed using polydT priming and α-[$^{33}$P]-dCTP. The efficiency of the reverse transcription was verified by measuring the radioactivity level of the purified cDNA. Hybridizations on the filters were carried out for 72 hours under the conditions described previously (26). After exposure on a phosphor screen high-resolution plate for 3 days, the image was scanned using a BAS 5000 phosphoimager and the spot intensities were acquired using the Array Gauge software (Fuji-Film™; Raytest, Paris la Defense, France). Any raw data with an intensity lower than the background plus three standard deviations were excluded. The normalization factors inter-assays were calculated with the global normalization method using the linear regression of all intensity data of the arrays, including the RNA standard intensities. For each arrayed gene, the resulting expression values, reported in arbitrary units, were compared to the expression value from the control cells. The relative changes in gene expression were analyzed using the Student's *t*-test ($p<0.05$).

*Gene expression:* The symbol (x) was used for gene transcripts with at least a two-fold increase, whereas the symbol (/) was used for gene transcripts with at least a two-fold decrease by TNFα or IFNγ.

*Protein expression analysis (translational activity).* Immunological methods can evaluate the effectiveness of TNFα and IFNγ at the protein level. Intracellular antigens, after fixation/permeabilization with IntraPrep™ Permeabilisation Reagent (Beckman Coulter) and membrane associated-antigens, were analyzed by indirect immunofluorescence. Specific monoclonal antibodies (anti-CD44, anti-thrombomodulin, anti-fibronectin, anti-cyclooxygenase-2) and appropriate conjugated isotypic controls were used before measuring antigen expression by flow cytometry (FACScalibur; Becton Dickinson, San Jose, CA, USA) (mean fluorescence intensity). MMP2 activities were determined using zymographic analysis, as already described (27).

## Results

*Biological responses.* In RT112 treated with TNFα (>10 ng/ml) for 48 hours, adherent cells were progressively detached, suggesting an apoptotic process. Flow cytometric analysis of the DNA cell content showed an increase in the percentage of subdiploid cells, thus confirming this apoptotic process in more than 30% of the cells. TNFα did not statistically modify the cell growth and did not induce an epithelial to mesenchymal transition (EMT) process (data not shown). RT112 cells

Table I. *Effects of TNFα±IFNγ on apoptosis.*

| Pretreatment | None | TNFα | IFNγ | TNFα+IFNγ |
|---|---|---|---|---|
| Treatment | | | | |
| None | 3%±1 | 6%±2 | 9%±2 | 30%±5 |
| TNFα+IFNγ | 23%±4 | 13%±4 | 15%±4 | 55%±6 |

RT112 cells were pretreated for 24 hours without (none) or with either TNFα (5 ng/ml) or IFNγ (1 ng/ml), or with TNFα+IFNγ (at 5 ng/ml and 1 ng/ml, respectively), washed and treated for 24 hours without inducer (none) or with inducers (20 ng/ml each TNFα+IFNγ). Cells were then treated with trypsin and used for determination of apoptosis using the Annexin V-FITC/PI method (Vybrant™ Apoptosis Assay kit). Data are the mean ± SD of three experiments.

reacted to IFNγ, showing an EMT and a growth inhibitory response. High doses of IFNγ (>10 ng/ml) induced an apoptotic process in 10–30% of the cells after 48 hours. When RT112 cells grew in the presence of both TNFα and IFNγ (15 ng/ml) for 24 hours, a synergistic effect was obtained: 80% of the cells died within an apoptotic process. However, when they were treated for 24–48 hours in the presence of either TNFα (5 ng/ml) or IFNγ (1 ng/ml), the RT112 cells acquired a resistance to the cytotoxic effect of TNFα+IFNγ (Table I). Therefore, TNFα and IFNγ showed an inverse dose-dependent effect: high doses induced an apoptotic process, whereas low doses induced a survival process.

*Genomic analysis in response to TNFα and IFNγ.* The biological responses of the RT112 cells to TNFα and IFNγ suggest that these cells could be an appropriate model system to study the signaling pathway mediated by the two cytokines. We therefore focused our investigations on the genes associated with the tumor progression characterized by an increase in cell survival, the induction of both angiogenesis and metastasis. To identify the genes transcriptionally regulated in the tumor cells after TNFα and IFNγ treatment, we used a DNA microarray consisting of 800 known genes. Among the genes modified by the two cytokines, many genes represented known TNFα-modulated genes (JUNB, RELB, IL-8, FN) or known IFNγ-modulated genes (B2M, STAT1, GBP1, u-PAR, IFNγR), providing independent validation of our system. Three categories of genes were identified: first, genes only modulated by TNFα (n=52); second, genes only modulated by IFNγ (n=66); and lastly, genes modulated by both TNFα and IFNγ (n=27). These genes modulated by TNFα and IFNγ in RT112 bladder tumor cells were distributed into five groups according to the ontological classification (http://www.geneontology.org) (Tables II and III).

Group I (apoptosis, survival and DNA repair): TNFα up-regulated eleven genes and down-regulated one gene, whereas IFNγ up-regulated nine genes and down-regulated one gene (Tables II and III). Exposure of RT112 cells to TNFα or IFNγ can either lead to apoptosis or to cell survival (see "Biological Responses"). Apoptosis is the result of a balance between the expression of pro-apoptotic and anti-apoptotic genes. Among the TNFα- and IFNγ-modulated genes, eight up-regulated genes induced an anti-apoptotic process and six up-regulated genes induced a pro-apoptotic process, whereas two down-regulated genes involved an anti-apoptotic process and one down-regulated gene a pro-apoptotic process. Consequently, the balance between apoptosis and survival suggests a post-transcriptional regulation.

Group II (cell proliferation-related genes): TNFα up-regulated nine genes, whereas IFNγ up-regulated seven genes and down-regulated three genes. Together, TNFα plus IFNγ up-regulated five genes. Only the CCNB2 gene was up-regulated by TNFα and down-regulated by IFNγ. The fact that the same pattern of genes was modulated by TNFα and IFNγ, whereas TNFα did not modify cell proliferation and IFNγ inhibited it, confirms the primordial role for a post-transcriptional regulation.

Group III (angiogenesis): Twelve out of thirteen genes modulated by the two cytokines were up-regulated, suggesting a vital role for TNFα and IFNγ in the angiogenic process.

Group IV (metastasis, migration and adhesion processes): TNFα up-regulated sixteen genes and down-regulated two genes, whereas IFNγ up-regulated fourteen genes and down-regulated one gene. Five genes were up-regulated by TNFα plus IFNγ. However, TNFα and IFNγ increased the same pattern of genes modulating the migration process, whereas only IFNγ-treated cells migrated. This confirms a role for post-transcriptional regulation.

Group V includes genes that have not yet been described in the tumor progression process. Among these genes, four of them, including MADH2, IFNGR2, CEBPD and IF141, were modulated by both TNFα and IFNγ.

*Post-transcriptional regulation.* To investigate this process, mRNA and protein expressions were analyzed in RT112 cells before and after TNFα and/or IFNγ induction.

Table IV shows MMP2, CD44 and THBD mRNAs and their protein expression. TNFα and IFNγ up-regulated both MMP2 mRNA (x3) and the amount of the intracellular MMP2 protein (x2 and x3.3, respectively). However, in the culture supernatant (CS), TNFα weakly increased secreted MMP2 protein, whereas IFNγ dramatically decreased it (x1.2 and x3.3, respectively). For THBD, IFNγ up-regulated both mRNA expression (x2.5) and the expression of its corresponding protein (thrombomodulin) (x1.8). For CD44, both TNFα and IFNγ up-regulated mRNA expression (x3), but TNFα did not modify CD44 protein expression, whereas IFNγ decreased it (x2).

In bladder cancer, cyclooxygenase-2 (cox-2) expression was enhanced in high-grade TCC, associated with both tumor invasiveness and poor patient outcome (28, 29). Moreover, cox-2 was regulated by type II phospholipase A2 (PLA2G2A

Table II. *TNFα-regulated genes identified by cDNA microarray technique.*

| GenBank accession no. | Gene name | Protein description | Untreated | TNFα | Fold increase |
|---|---|---|---|---|---|
| Apoptosis and survival | | | | | |
| NM002657 | PLAGL2 | pleiomorphic adenoma gene link 2 | 16 | 8 | / 2 |
| AK027071 | TSC22 | TGFβ stimulated protein TSC22 | 4 | 15 | x 4 |
| NM002970 | SAT | spermidine/spermine acetyltransferase | 3 | 7 | x 2.3 |
| NM001753 | CAV1 | caveolin alpha | 2 | 8 | x 4 |
| NM002228 | JUN | jun | 3 | 6 | x 2.3 |
| NM002648 | PIM1 | pim-1 oncogene | 20 | 45 | x 2.5 |
| M10943 | MT1F | metallothionein | 5 | 15 | x 3 |
| NM001013 | RPS9 | ribosomal protein 9 | 1 | 5 | x 5 |
| NM006509 | RELB | part of NF-KB | 6 | 16 | x 2.5 |
| NM002412 | MGMT | O-6-methylguanine-DNA metyltransferase | 1 | 4 | x 4 |
| NM000832 | GSTP1 | glutathione transferase P1 | 45 | 90 | x 2 |
| NM001320 | CSNK2B | casein kinase 2B | 3 | 6 | x 2 |
| Cell proliferation | | | | | |
| NM000075 | CDK4 | cdk4 | 2 | 4 | x 2 |
| NM000389 | CDKN1A | p21/waf1 | 12 | 38 | x 3.1 |
| L24498 | GADD45A | DNA damage inducible protein gadd45 | 1,5 | 5 | x 3.5 |
| NM004701 | CCNB2 | cyclin B2 | 3 | 7 | x 2.3 |
| NM004526 | MCM2 | cdc19p | 1 | 3 | x 3 |
| BC001854 | MAT2A | methionine adenyltransferase IIA | 4 | 8 | x 2 |
| AF090094 | OAZ1 | ornithine decarboxylase antizyme 1 | 5 | 12 | x 2.5 |
| NM006185 | NUMA1 | nuclear mitotic apparatus protein 1 | 2.5 | 5 | x 2 |
| NM06378 | SEMA4D | semaphorine 4D/CD100 | 1 | 4 | x4 |
| Angiogenesis | | | | | |
| NM000584 | IL8 | interleukin-8 | 2 | 23 | x 12 |
| NM002165 | ID1 | zing finger protein id1 | 30 | 60 | x 2 |
| NM002167 | ID3 | zing finger protein id3 | 6 | 18 | x 3 |
| NM002607 | PDGFA | PDGFalpha | 4 | 8 | x 2 |
| NM001530 | HIF1A | hypoxia inducible factor 1 | 2 | 8 | x 4 |
| NM002994 | SCYB5 | chemokine | 1 | 5 | x 5 |
| AF022375 | VEGF | VEGF | 5 | 10 | x 2 |
| NM012215 | MGEA5 | hyaluronidase | 1 | 4 | x 4 |
| NM000300 | PLA2G2A | phospholipase A2 | 1 | 3 | x 3 |
| Metastatic process | | | | | |
| NM002525 | NRD1 | nardilysin | 1 | 1 | x 7 |
| NM002210 | ITGAV | integrin alpha 6/CD51 | 1 | 5 | x 5 |
| NM002205 | ITGB5 | integrin beta 5 | 3 | 7 | x 2.3 |
| NM014288 | ITGB3BP | integrin beta 3 binding protein | 1 | 3 | x 3 |
| NM004753 | SDC1 | syndecan 1 | 1 | 3 | x 3 |
| NM003255 | TIMP2 | TIMP2 | 1 | 3 | x 3 |
| NM004530 | MMP2 | MMP2 | 1 | 3 | x 3 |
| NM002427 | MMP13 | MMP13 | 1 | 10 | x 10 |
| NM001814 | CTSC | cathepsin C | 1 | 12 | x 12 |
| NM001912 | CTSL | cathepsin L | 1 | 6 | x 6 |
| AJ251595 | CD44 | CD44 antigen | 1 | 3 | x 3 |
| NM002906 | RDX | radixin (ERM family/ligand of CD44) | 2 | 1 | / 2 |
| NM002996 | SCYD1 | fractalkin | 1 | 5 | x 5 |
| NM032466 | ASPH | aspartate beta hydroxylase | 2 | 1 | / 2 |
| NM014360 | CDH1 | E-cadherin | 3 | 10 | x 3 |
| NM001456 | FLNA | filamin alpha | 8 | 19 | x 2.5 |
| NM001355 | DDT | D-dopachrome tautomerase | 5 | 10 | x 2 |
| NM001780 | CD63 | CD63 antigen | 25 | 53 | x 2.1 |

*continued on the next page*

Table II *continued*

| GenBank accession no. | Gene name | Protein description | Untreated | TNFα | Fold increase |
|---|---|---|---|---|---|
| Other genes | | | | | |
| NM005901 | MADH2 | Smad2 | 4 | 1 | / 4 |
| NM005534 | IFNGR2 | Interferon gamma receptor 2 | 3 | 10 | x 3 |
| NM005195 | CEBPD | KIAA0146 protein | 3 | 15 | x 5 |
| NM002055 | GFAP | GFAP | 1 | 2.5 | x 2.5 |
| NM004148 | NINJ1 | ninjurin 1 | 2 | 6 | x 3 |
| NM055027 | CARM1 | coactivator-associated arginine methyltr. | 1 | 3 | x 3 |
| NM001068 | TOP2B | topoisomerase 2B | 1 | 3 | x 3 |
| L 22342 | IF141 | interferon-induced protein 41 | 2 | 6 | x 3 |
| NM001945 | DTR | Diphtheria toxin receptor | 4 | 18 | x 4 |

Four subgroups were identified in relation to bladder tumor progression (chemoresistance and anti-apoptotic process, cell proliferation, angiogenesis and metastatic process) and one subgroup without relation. The symbols (x and /) used in this table are defined in the Materials and Methods section.

gene) (30). In our experiments, both TNFα and IFNγ up-regulated PLA2G2A gene expression (x3). However, immunological analysis (FACS) showed that IFNγ increased both the percentage of cox-2-positive cells from 6%±2 to 90%±10 and the cox-2 antigen expression from 12 to 21 (arbitrary units), whereas TNFα did not statistically modify it.

Finally, TNFα and IFNγ up-regulated IFNGR2 mRNA expression (x3 and x2, respectively) and FACS analysis showed an increase in the IFNγ-R antigen expression from 35±5 to 72±12 (TNFα) and from 35±5 to 53±7 (IFNγ) (FACS arbitrary units). All these results suggest a post-transcriptional activity.

*Up-regulated genes associated with poor outcome in bladder cancer.* In bladder cancer, several studies identified critical molecular targets (genes and proteins) altered during tumor progression and associated with poor patient outcome. We identified some genes modulated by either TNFα or IFNγ that were overexpressed in bladder cancer (Table V). Among these genes, ten were up-regulated by both TNFα and IFNγ, suggesting that these cytokines may induce the tumor progression process in bladder cancer using a similar pathway.

## Discussion

The concept of a metastatic cell population suggests a selective competition favoring the survival of a subpopulation (31). In bladder cancer, the dominance of the subpopulation of metastatic tumor cells that can appear during BCG treatment (the most potent therapy currently used in this pathology) requires activation of a set of effector genes (32, 33). Because intravesical BCG therapy is followed by the sequential appearance of transient high levels of IFNγ and TNFα in the urine of these patients, the effects of the two cytokines were investigated on RT112 bladder tumor cells (6, 34).

In this study, the RT112 cells were cultured in the presence of variable doses of TNFα or IFNγ The biological response (growth, apoptosis and chemoresistance) and the gene and protein expression were then analyzed using a DNA microarray approach and immunological methods. High doses of either TNFα or IFNγ (>10 ng/ml) for 48 hours induced an apoptotic cell death in 10–30% of the RT112 cells, whereas low doses of TNFα (<5 ng/ml) or IFNγ (<1 ng/ml) induced the development of resistance to the cytotoxic effect of the high doses of both TNFα and IFNγ (11 and Table I). Low doses of IFNγ also induced an epithelial to mesenchymatous transformation (EMT) (11), confirming that IFNγ induced morphological changes as well as cytostatic and cytotoxic processes, whereas TNFα only induced a cytotoxic process (11, 35). Following cultures in the presence of TNFα or IFNγ, the global patterns of transcriptional activation obtained with the transcriptomic approach provided essential information regarding cell response to the bladder tumor progression process (Tables II and III). The most striking feature of gene activation by TNFα was the massive induction of IL-8 (x12), MMP-13 (x10), CTSL (x6), GBP1 (x13) and NDR1 (x7) gene transcription. In bladder cancer, IL-8, MMP-13, CTSL and GBP1 controlled the metastatic process (36-39).

Both TNFα and IFNγ increased IL-8 and VEGF mRNA expression, which represent the divergent developmental pathways in the pathogenesis of bladder carcinoma (40). IL-8 appeared to be up-regulated in early precursor lesions (CIS), whereas VEGF appeared to be up-regulated at later stages (muscle invasion) by increasing RELB gene expression. This component of the NF-Î B transcription factor was frequently overexpressed in bladder cancer and inhibited the activity of some chemotherapeutic agents (41). TNFα and IFNγ also increased PLA2G2A gene expression. On the other hand, its related protein (phospholipase A2) amplified TNFα-stimulated prostaglandin E2 (PGE2), which stimulated cox-2,

Table III. *IFNγ-regulated genes identified by cDNA microarray technique.*

| GenBank accession no. | Gene name | Protein description | Untreated | IFNγ | Fold increase |
|---|---|---|---|---|---|
| Apoptosis and survival | | | | | |
| NM002831 | PTPN6 | protein tyrosine phosphatase | 5 | 10 | x 2 |
| AK027071 | TSC22* | TGFβ stimulated protein TSC22 | 4 | 10 | x 2.5 |
| NM002970 | SAT* | spermidine acetyl transferase | 3 | 8 | x 2.6 |
| NM002228 | JUN* | JUN | 3 | 8 | x 2.5 |
| NM003225 | TFF1 | trefoil factor 1 | 2 | 5 | x 2.5 |
| NM001912 | MCL1 | BCL2 related protein | 1 | 3 | x 3 |
| NM002648 | PIM1* | protein peptidyl-prolylcis/trans isomerase | 20 | 48 | x 2.5 |
| NM005346 | HSPA1B | Heat shock protein 1A | 8 | 4 | / 2 |
| NM006509 | RELB* | part of NF-KB | 6 | 13 | x 2 |
| NM001320 | CSNK2B* | casein kinase 2B | 3 | 15 | x 5 |
| Cell proliferation | | | | | |
| NM000075 | CDK4* | CDK4 | 2 | 7 | x 3.2 |
| NM007315 | STAT1 | STAT1 | 1 | 6 | x 6 |
| NM000389 | CDKN1A* | CDK1A/p21 | 12 | 27 | x 2.3 |
| L24298 | GADD45A* | DNA damage inducible protein gadd45 | 1,5 | 4 | x 2.5 |
| NM004701 | CCNB2* | cyclin B2 | 3 | 1 | / 3 |
| NM013283 | MAT2B | methionine adenyltransferase IIB | 5 | 1 | / 5 |
| NM001255 | CDC20 | CDC20 | 8 | 3 | / 2.5 |
| NM2945 | RPA1 | replication protein A1 | 2 | 6 | x 3 |
| NM001604 | PAX6 | homeotic protein aniridia | 7 | 19 | x 3 |
| AF090904 | OAZ1* | ornithine decarboxylase antizyme 1 | 5 | 10 | x 2 |
| Angiogenesis | | | | | |
| NM000584 | IL8* | interleukin-8 | 2 | 4 | x 2 |
| NM003680 | YARS | tyrosyl tRNA synthetase | 3 | 7 | x 2.3 |
| NM002165 | ID1* | zing finger protein | 30 | 70 | x 2.3 |
| NM002825 | PTN | pleiotrophin | 15 | 32 | x 2.1 |
| AF022375 | VEGF* | VEGF | 5 | 15 | x 3 |
| NM012215 | MGEA5* | hyaluronidase | 1 | 4 | x 4 |
| NM002053 | GBP1 | guanylate binding protein-1 | 1 | 13 | x 13 |
| NM000300 | PLA2G2A* | phospholipase A2 | 1 | 3 | x 3 |
| Metastatic process | | | | | |
| NM003005 | SELP | P selectin /CD62 | 12 | 27 | x 2.2 |
| NM004530 | MMP2* | MMP2 | 1 | 3 | x 3 |
| NM001814 | CTSC* | cathepsin C | 1 | 9 | x 9 |
| NM001912 | CTSL* | cathepsin L | 1 | 3 | x 3 |
| NM002859 | PXN | paxillin | 4 | 11 | x 2.8 |
| NM004360 | CDH1* | E-cadherin | 3 | 16 | x 5.2 |
| NM032466 | ASPH | aspartate beta hydroxylase | 2 | 4 | x 2 |
| NM001848 | COL6A1 | collagen VI alpha | 1 | 3 | x 3 |
| NM001845 | COL4A1 | collagen IV alpha | 6 | 1 | / 6 |
| NM002996 | SCYD1* | fractalkin | 1 | 3 | x 3 |
| NM005310 | GRB7 | growth factor R band binding protein | 4 | 12 | x 3 |
| NM000210 | ITGAV* | integrin alpha 6/CD51 | 4 | 8 | x 2 |
| NM004034 | ANXA7* | annexin 7 | 1 | 3 | x 3 |
| NM001355 | DDT* | D-dopachrome tautomerase | 5 | 13 | x 2 |
| AJ251595 | CD44* | CD44 antigen | 1 | 3 | x 3 |
| Other genes | | | | | |
| NM005901 | MADH2* | Smad2 | 4 | 1.5 | / 3 |
| NM091106 | RGS3 | regulator of G-protein signaling 3 | 4 | 10 | x 2.5 |

*continued on the next page*

Table III *continued*

| GenBank accession no. | Gene name | Protein description | Untreated | IFNγ | Fold increase |
|---|---|---|---|---|---|
| NM001958 | EEF1A2 | eukaryotic translation elongation factor 1 | 20 | 57 | x 2.8 |
| M10943 | MT1F | metallothionein 1F | 5 | 18 | x 3. 5 |
| NM004103 | PTK2B | protein tyrosine kinase 2B | 4 | 9 | x 2 |
| NM014302 | SEC61G | sec61 gamma | 2 | 6 | x 3 |
| NM004753 | SDR1 | short-chain dehydrogenase/reductase 1 | 2 | 5 | x 2.5 |
| NM005534 | IFNGR2* | interferon gamma receptor 2 | 3 | 6 | x 3 |
| NM005195 | CEBPD* | KIAA0146 protein | 2 | 10 | x 5 |
| NM004048 | B2M | Beta 2 microglobulin | 22 | 250 | x 12 |
| NM004355 | CD74 | CD74 antigen | 1 | 3 | x 3 |
| NM002038 | G1P3 | interferon alpha inducible protein | 1 | 4 | x 4 |
| NM005101 | ISG15 | interferon-stimulated protein, 15kDa | 1 | 12 | x 12 |
| NM 003418 | ZNF9 | zing finger protein 9 | 10 | 20 | x 2 |
| L 22342 | IF141* | interferon-induced protein 41 | 1 | 4 | x 4 |

Legends: see Table II. *: Gene up-regulated by TNFα and IFNγ.

playing a role in the development and invasion of bladder cancer (42). TNFα and IFNγ also increased CSNK2B (x2 and x5, respectively), and its related protein, casein kinase 2B, can play an anti-apoptotic role in tumorigenesis (43).

TNFα or IFNγ also induce the expression of a great number of genes that are essential regulators of the cell cycle (CDK4, CDKN1A, CDC20, CCNB2). As a result, cyclin B2 (CCNB2) associated with cdc2 forms an active cyclin B/cdc2 complex necessary for G2/M progression. Moreover, cdk4 binds to cyclin D, forming an active complex necessary for G1/S progression that can be inhibited by p21/waf1 (CDKN1A). Interestingly, in our experiments, IFNγ increased CDKN1A and decreased both CCNB2 and CDC20. These observations correlated with the fact that IFNγ inhibited RT112 cell growth. However, the increase in CDK4 gene expression, with peak expression in the G2- and M-phases of the cell cycle, was also detected in both TNFα- and IFNγ-treated cells. Moreover, several transcription factors that bind to the CDKN1A promoter region have been identified including p53, STAT and CEBP. STAT and CEBP were increased, whereas p53 was not, indicating that CDKN1A could be induced independently of p53. It is also possible that the increase in CDKN1A could be due either to the post-transcriptional regulation or to the stabilization of the mRNA (44). All these results suggest that low doses of both TNFα and IFNγ modulated mRNA expression of the tumor marker. This could explain both the tumor progression and the short survival of bladder cancer patients with low response to BCG therapy (45).

As well as the detection of genes involved in bladder tumor progression, the correlation of the mRNA expressions with the corresponding protein have been investigated. This was studied using either immunological (protein expression) or zymographic analysis (protein activity). For the tested molecules, mRNA and protein levels did not correlate in all experiments. Our results corroborate those already published (46, 47). Anderson *et al.* showed a relatively poor correlation between mRNA and protein levels in human liver (R=0.48) (46). However Verhoeckx *et al.* found a correlation coefficient of 0.86 in the U937 cell line growing in the presence of phorbol ester (PMA) (47). Nevertheless, this correlation coefficient of 0.86, calculated according to the method of Anderson *et al.,* is biased by highly abundant proteins and mRNAs. So, omitting this bias, Verhoeckx found a correlation coefficient of 0.49, which is similar to previously reported results. However, our results did not corroborate those already published on bladder carcinoma (48). These discrepancies could be explained by mRNA expression being constitutive in the study of Orntoft *et al.* In our experiments, mRNA expression was induced due to the effect of TNFα or IFNγ on the RT112 cells. Therefore, we hypothesized that, in the bladder tumor, the post-transcriptional regulation is functional in low-grade tumors, suggesting that defects in the translation process could be one of the keys of bladder tumorigenesis.

In conclusion, this study showed that, in bladder cancer cells, low doses of both TNFα and IFNγ may increase the expression of mRNAs associated with the tumor progression process. However, there was no correlation with protein expression, suggesting that the non-invasive bladder tumor cells can modulate the protein synthesis induced by exogenously added cytokines. Taken together, these results suggest a vital role for post-transcriptional regulation. To confirm this, a proteomic analysis should be associated with a cDNA microarray, and thus these new technologies will take a central place in the understanding, diagnosis, prognosis and treatment of bladder cancer.

Table IV. *mRNA and protein expression.*

| | | | Treatments | | |
|---|---|---|---|---|---|
| Name | Expression | | Control | TNFα | IFNγ |
| CD44 | mRNA | | 1 | 3 | 3 |
| | protein | PPC | 100% | 90%±5 | 70%±5 |
| | | MFI | 33±2 | 30±3 | 18±3 |
| MMP2 | mRNA | | ND | 3 | 3 |
| | protein | IC | 91±2 | 194±7 | 303±23 |
| | | CS | 1680±10 | 2093±12 | 753±13 |
| THBD | mRNA | | 6 | 9 (NS) | 16 |
| | protein | PPC | 100% | 100% | 100% |
| | | MFI | 20±4 | 23±3 | 35±5 |

RT112 cells were cultured without (control) or with inducer (TNFα or IFNγ) (5ng/ml) for 24 hours (mRNA analysis) or 48 hours (protein analysis). mRNAs were extracted as described in Materials and Methods and analyzed using cDNA microarray. Proteins were analyzed using monoclonal antibodies directed against CD44, fibronectin (FN) or thrombomodulin (THBD), and their antigene expression was quantified using FACS. Data are the results of two experiments. PPC: % of positive cells; MFI: mean fluorescence intensity, IC: intracellular (pg/mg of protein), CS: culture supernatant (pg/ml/$10^6$ cells/24h); NS : non significant variation. ND: undetectable.

Table V. *Genes up-regulated by TNFα and/or IFNγ and overexpressed in bladder cancer.*

| |
|---|
| ITGA5 (x5 and x2)* |
| THBD (x 2) (IFNγ) |
| IL8 (x12 and x2)* |
| MT1F (x 3)(TNFα) |
| VEGF (x 2 and x 3)* |
| MGEA5 (x 4)* |
| RELB (x 2.5 and x 2)* |
| TIMP2 (x 3)(TNFα) |
| MMP2 (x 3)(TNFα) |
| MMP13 (x 10)(TNFα) |
| CTSL (x 6 and x 3)* |
| CDH1 (x 3 and x 5)* |
| CTSC (x 12 and x 9)* |
| CSNK2B (x 2 and x 5)* |
| DTR (x 4 )(TNFα) |
| CDKN1A (x 3 and x 2)* |

Genes modulated by TNFα only (TNFα), by IFNγ only (IFNγ) or by the two cytokines (*), overexpressed in bladder tumor and associated with a poor outcome in bladder cancer patients.

## Acknowledgements

This research was supported by grants from the Direction de la Recherche Clinique (DRC) of the Centre Hospitalier Universitaire de Grenoble (France). We express our thanks to Linda Northrup for her help in English language editing and to Trocme Candice for MMP2 experimentation.

## References

1 Morales A, Eidinger D and Bruce AW: Intracavity bacillus Calmette Guerin in the treatment of superficial bladder tumors. J Urol *116*: 180-186, 1976.

2 Herr H, Laudone VP and Badalement RA: Bacillus Calmette Guerin therapy alters the progression of superficial bladder cancer. J Clin Oncol *6*: 1450-1455, 1988.

3 Patard JJ, Saint F, Velotti F, Abbou CC and Chopin DK: Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review. Urol Res *26*: 155-159, 1998.

4 Prescott S, James K and Hargreave TB: Radiommunoassay detection of interferon-gamma in urine after intravesical BCG therapy. J Urol *144*: 1248-1252, 1990.

5 De Boer EC, De Jong WH and Steerenberg PA: Induction of interleukins-1 (IL-1), IL-2, IL-6 and tumor necrosis factor during intravesical immunotherapy with bacillus Calmette Guerin in superficial bladder cancer. Cancer Immunol Immunoth *34*: 306-311, 1992.

6 Park DS, Park JH, Lee JL, Lee JM, Kim YH, Lee JM and Kim SJ: Induction of ICAM-1, HLA-DR molecules by IFN-gamma and oncogene expression in human bladder cancer cell lines. Urol Int *59*: 72-80, 1997.

7 Hawkyard SJ, Jackson AM, James K, Prescott S, Smyth JF and Chisholm GD: The inhibitory effects of interferon gamma on the growth of bladder cancer cells. J Urol *147*: 1399-1403, 1992.

8 Stavropoulos NE, Hastazeris K, Filiadis I, Milhailidis I, Ioachim E, Liamis Z and Kalomiris P: Intravesical instillation of interferon gamma in the prophylaxis of high risk superficial bladder cancer. Scand J Urol Nephrol *36*: 218-222, 2001.

9 Giannopoulos A, Constantinides C, Fokaeas E, Stravodimos C, Giannopoulos M, Kyroudi A and Gounaris A: The immunomodulating effect of interferon-γ intravesical instillations in preventing bladder cancer recurrence. Clin Cancer Res *9*: 5550-5558, 2003.

10 Kelly SA, Gschmeissner S, East N and Balkwill FR: Enhancement of metastatic potential by γ-interferon. Cancer Res *51*: 4020-4027, 1991.

11 Champelovier P, Simon A, Garrel C, Levacher G, Praloran V and Seigneurin D: Is interferon γ one key of metastatic potential increase in human bladder carcinoma ? Clin Cancer Res *9*: 4562-4569, 2003.

12 Matsuura K, Otsuka F and Fujisawa H: Effects of interferons on tumor necrosis factor production from human keratinocytes. Cytokine *10*: 500-505, 1998.

13 Masafumi T, Yum K and Vilcek J: Interferon-γ enhances expression of cellular receptors for tumor necrosis factor. J Immunol *136*: 2441-2444, 1996.

14 Fiers W: Tumor necrosis factor: Characterization at the molecular, cellular and *in vivo* level. FEBS Lett *285*: 199-212, 1991.

15 Himeno T, Watanabe N, Yamauchi N, Maeda M, Tsuji Y, Okamoto T, Neda H and Niitsu Y: Expression of endogenous tumor necrosis factor as a protective protein against the cytotoxicity of exogenous tumor necrosis factor. Cancer Res *50*: 4941-4945, 1990.

16 Beg AA and Baltimore D: An essential role for NF-kB in preventing TNF-α-induced cell death. Science *274*: 782-784, 1996.

17 Ito H, Kyo S, Kanaya T, Takakura M, Koshida K, Namiki M and Inoue M: Detection of human telomerase reverse transcriptase messenger RNA in voided urine samples as a useful diagnostic tool for bladder cancer. Clin Cancer Res *4*: 2807-2810, 1998.

18 Thykjaer T, Workman C, Kruhoffer M, Demtroder K, Wolf H, Andersen LD, Frederiksen CM, Knudsen S and Orntoft TF: Identification of gene expression patterns in superficial and invasive human bladder cancer. Cancer Res *61*: 2492-2499, 2001.
19 Sanchez-Carbayo M, Socci ND, Charytonowicz E, Lu M, Prystowsky M, Childs G and Cordon-Cardo C: Molecular profiling of bladder cancer using cDNA microarrays : defining histogenesis and biological phenotypes. Cancer Res *62*: 6973-6980, 2002.
20 Dyrskot L, Thykjaer T, Kruhoffer M, Jensen JL, Marcussen N, Hamilton-Dutoit S, Wolf H and Orntoft TF: Identifying distinct classes of bladder carcinoma using microarrays. Nature Genetics *33*: 90-96, 2003.
21 Jansen BJH, van Ruissen F, de Jongh G, Zeeuwen PLJM and Schalkwijk J: Serial analysis of gene expression in differentiated cultures of human epidermal keratinocytes. J Invest Dermatol *116*: 12-22, 2001.
22 Gallagher J, Howlin J, McCarthy C, Murphy EP, Bresnihan B, Fitzgerald O, Godson C, Brady HR and Martin F: Identification of Naf1/ABIN-1 among TNF-α-induced expressed genes in human synoviocytes using oligonucleotide microarrays. FEBS Lett *551*: 8-12, 2003.
23 Der SD, Zhou A, Williams BRG and Silverman RH: Identification of genes differentially regulated by interferon α, β or γ using oligonucleotid arrays. Proc Natl Acad Sci USA *95*: 15623-15628, 1998.
24 Patrone L, Damore MA, Lee MB, Malone CS and Wall R: Genes expressed during the IFNγ-induced maturation of pre-B cells. Mol Immunol *38*: 597-606, 2001.
25 El Atifi M, Dupré I, Rostaing B, Benabid AL and Berger F: Quantification of DNA probes on nylon microarrays using T4 polynucleotide kinase labeling. BioTechniques *35*: 262-266, 2003.
26 El Atifi M, Dupré I, Rostaing B, Chambaz EM, Benabid AL and Berger F: Long oligonucleotide arrays on nylon for large-scale gene expression analysis. BioTechniques *33*: 612-618, 2002.
27 Monier F, Mollier S, Guillot M, Rambeaud JJ, Morel F and Zaoui P: Urinary release of 72 and 92 kDa gelatinases, TIMPs, N-GAL and conventional prognostic factors in urothelial carcinomas. Eur Urol *42*: 356-363, 2002.
28 Shirahama T, Arima J-I, Akiba S and Sakakura C: Relationship between cyclooxygenase-2 expression and tumor invasiveness and patient survival in transitional cell carcinoma of the urinary bladder. Cancer *92*: 188-193, 2001.
29 Shariat S, Matsumoto K, Kim J, Ayala GE, Zhou J-H, Jian W, Benedict WF and Lerner SP: Correlation of cyclooxygenase-2 expression with molecular markers, pathological features and clinical outcome of transitional cell carcinoma of the bladder. J Urol *170*: 985-989, 2003.
30 Bidgood MJ, Jamal OS, Cunningham AM, Brooks PM and Scott KF: Type IIA secretory phospholypase A2 up-regulates cycloo-xygenase-2 and amplifies cytokine-mediated prostaglandin production in human rheumatoid synoviocytes. J Immunol *165*: 2790-2797, 2000.
31 Fidler IJ and Hart IR: Biologic diversity in metastatic neoplasm-origins and implications. Science (Washington DC) *217*: 998-1001, 1982.
32 Herr HW: Progression of stage T1 bladder tumors after intravesical bacillus Calmette-Guerin. J Urol *145*: 40-45, 1991.
33 Sanchez-Carbayo M, Socci ND, Lozano JJ, Li W, Charytonowicz E, Belbin TJ, Prystowsky M, Ortiz AR, Childs G and Cordon-Cardo C: Gene discovery in bladder cancer progression using cDNA microarray. Am J Pathol *163*: 505-516, 2003.
34 Balbay D, Ozen H, Ozkardes H, Barut A, Bakkaloglu M, Tasar C and Remzi D: Detection of urinary interleukin-2, interleukin-2 receptor, and tumor necrosis factor levels in patients with superficial bladder tumors after intravesical BCG immunotherapy. Urology *43*: 187-190, 1994.
35 Hawkyard SJ, Jackson AM, James K, Prescott S, Smyth JF and Chisholm GD: The inhibitory effects of interferon gamma on the growth of bladder cancer cells. J Urol *147*: 1399-1403, 1992.
36 Inoue K, Slaton JW, Kim SJ, Perrotte P, Eve BY, Bar-Eli M, Radin R and Dinney CP: Interleukin 8 expression regulates tumorigenesis and metastasis in human bladder cancer. Cancer Res *60*: 2290-2299, 2000.
37 Guenzi E, Topolt K, Luberseder-Martellato C, Jorg A, Naschberger E, Benelli R, Albini A and Sturzl M: The guanylate binding protein-1 GTPase controls the invasive and angiogenic capability of endothelial cells through inhibition of MMP-1 expression. EMBO J *22*: 3772-3782, 2003.
38 Bostom PJ, Ravanti L, Reunanen V, Soderstrom KO, Kahari VM and Laato M: Expression of collagenase-3 (matrix metalloproteinase-13) in transitional-cell carcinoma of the urinary bladder. Int J Cancer *88*: 417-423, 2000.
39 Staack A, Koenig F, Daniltchenko D, Hauptmann S, Loening SA, Schnorr D and Jung K: Cathepsins B, H, and L activities in urine of patients with transitional cell carcinoma of the bladder. Urology *59*: 308-312, 2002.
40 Izawa JI, Slaton JW, Kedar D, Karashima T, Perrotte P, Czerniak B, Grossman HB and Dinney CP: Differential expression of progression-related genes in the evolution of superficial to invasive transitional cell carcinoma of the bladder. Oncol Rep *8*: 9-15, 2001.
41 Xie DH, Tang XD, Xia SJ, Tan JM, Wang XH and Cai Y: Expression of NF-kappa B in human bladder cancer and its clinical significance. Ai Zheng *21*: 663-667, 2002.
42 Shirahama T, Arima J, Akiba S and Sakakura C: Relation between cyclooxygenase-2 expression and tumor invasiveness and patient survival in transitional cell carcinoma of the bladder. Cancer *92*: 188-193, 2001.
43 Litchfield DW: Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. Biochemical J *369*: 1-15, 2003.
44 Park J-W, Jang M-A, Lee YH, Passanniti A and Kwon TK: p53-independent elevation of p21 expression by PMA results from PKC-mediated mRNA stabilization. Biochem Biophysic Res Com *280*: 244-248, 2001.
45 Patard JJ, Saint F, Velotti F, Abbou CC and Chopin DK: Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review. Urol Res *26*: 155-159, 1998.
46 Anderson L and Seilhaer J: A comparison of selected mRNA and protein abundances in human liver. Electrophoresis *18*: 533-537, 1997.
47 Verhoeckx K, Bijlsma S, de Groene E, Witkamp R, van der Greef J and Rodenburg R: A combination of proteomics, principal component analysis and transcriptomics is a powerful tool for the identification of biomarkers for macrophage maturation in the U937 cell line. Proteomics *4*: 1014-1028, 2004.
48 Orntoft TF, Thykjaer T, Waldman FM, Wolf H and Celis JE: Genome-wide study of gene copy numbers, transcripts, and protein levels in pairs of non-invasive and invasive human transitional cell carcinomas. Mol Cell Proteomics *1*: 37-45, 2002.

*Received November 16, 2004*
*Accepted November 29, 2004*