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Molecular Analysis of Xenograft Models of Human Cancer Cachexia – Possibilities for Therapeutic Intervention

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Abstract. *Approximately 50% of all cancer patients develop cachexia, a paraneoplastic syndrome that is characterized by wasting of adipose tissue and skeletal muscle mass. Cytokines, including TNF-α, interleukins-1, -6, and interferon-γ are known mediators of the cachectic process. The latter however represent only one of many imbalanced systems in cancer cachexia. The aim of this study was to further delineate the pathogenesis of cachexia by molecular profiling. Human renal cancer xenografts that do and do not induce cachexia in mice were used as disease models. Cachexia-associated gene expression was studied on Human Genome U95 Affymetrix arrays and revealed several new genes such as TNF-α ligand superfamily protein, interferon-γ treatment inducible protein, and DKFZ5641I1922. The expression of the IL-8 gene was also elevated in cachexia inducing xenografts (CIX). At the protein level, TNF-α was found expressed only in CIX, whereas IL-1 and IL-6 were not cachexia specific. Levels of parathyroid hormone-related protein were elevated in CIX and accompanied by hypercalcemia. COX-2 and prostaglandin E2 were also found to be over expressed. By using the COX-2 inhibitors rofecoxib and nimesulide, we were able to delay tumor-mediated wasting in vivo. Overall, our results suggest that cachexia is a multigenetic disease that will require complex combinations of drugs for an effective therapeutic intervention.*

Cancer is frequently associated with anorexia, progressive weight loss and accelerated malnutrition resulting in the depletion of whole body lipid and protein stores. This body wasting condition, named cachexia, is accompanied by poor quality of life, poor response to chemotherapy and reduced survival time irrespective of tumor mass or the presence of metastases (1). The pathogenesis of cancer-associated cachexia is not fully understood, however, multiple mediators including neuroendocrine hormones, tumor-specific factors and pro-inflammatory cytokines may be involved (2-4). Although experimental evidence strongly supports a role of the pro-inflammatory cytokines TNF-α, interleukin-1 (IL-1) and interleukin-6 (IL-6) in the etiology of cachexia, a causative function for cytokines in cachexia development remains controversial (5). While a clear association between induction of cachexia and cancer exists for TNF-α (also termed cachexin), IL-1 appears not to be necessary for inducing cachexia, and IL-6 is not active on its own, but only in concert with other cachectic factors (6). Prostaglandins, particularly prostaglandin E2 (PGE2), are key down-stream effecter molecules of cytokine activity. Inhibitors of the PGE2-signalling pathway exist and have been used to prove the involvement of cytokines in cachexia development. Thus, by blocking PGE2 activity with specific cycloxygenase (COX) inhibitors, responses were noted in tumor-bearing mice. Anorexia, cachexia and even tumor progression were markedly attenuated in murine tumor models of cachexia with meloxicam and indometacin. As a result, a clinical trial in patients with advanced cancer was initiated using indometacin (7, 8). Anti-TNF-α antibodies were also studied, but with only limited success and were not further pursued (9). More recently treatment with IGF-1, owing to its prominent role in muscle metabolism, has been proposed as a promising approach for the prevention of cancer-related muscle atrophy (4, 10). However, targeted treatments remain experimental at best and current therapies aiming to alleviating cancer cachexia are mostly based on nutritional approaches. Hence, a better knowledge of the molecular pathways governing cachexia is required in order to develop more effective treatments.

In this study we examined the role of cytokines and novel factors that may play a role in the development of cancer cachexia. Three CIXs (RXF 393, RXF 486, RXF 1220) that induce cachexia in nude mice independent of tumor growth

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and size, and two xenografts (RXF 631 and RXF 944LX) that do not induce any weight loss (non-CIX) were selected from a collection of renal (RXF) cell cancer xenograft models that were established from patient tumors at the University of Freiburg. From the latter we were also able to generate permanent cell lines which show the same features as the original xenografts. These model systems enabled us to study and compare the molecular biology of cachexia-inducing tumors *versus* those that do not induce cachexia *in vitro* and *in vivo*.

Materials and Methods

Animals. Our in house outbred nude mouse strain of NMRI genetic background (20-22 g, 4-6 weeks of age) was used for all experiments. The animals were housed in Macrolon cages under natural day light cycles and maintained at ambient temperature of 24±1˚C. Mice were fed with standard chow (Altromin, Lage) and water *ad libitum*. All animal experiments were approved by the Regierungspräsidium Freiburg and studies essentially conducted following the UKCCR guidelines for the use of animals in experimental neoplasia (11).

Human tumor xenografts. Explants of patient-derived renal cell carcinomas (RXF) were established in nude mice by subcutaneous transplantation into both flanks *s.c.* as described by Fiebig *et al.* (12). When implanted *s.c.*, the tumor grew locally with a reproducible growth pattern. Tumor tissues were collected when tumors reached a volume of 0.7-1.5 cm^3 and pieces from the viable rim were shock-frozen in liquid nitrogen for preparation of protein lysates and RNA for Affymetrix array analysis or RT-PCR experiments. A part of each tumor was further fixed in 10% PBS buffered formaldehyde and then embedded in paraffin for immunohistochemical analysis.

Cell culture. Permanent renal cell cancer cell lines were cultured in RPMI 1640 medium supplemented with L-glutamine and 10% heat deactivated fetal calf serum and 1 μg/ml gentamycin. Primary cultures were used at the passages 1-20 after explanting tumor tissue from nude mice. They were cultured in ISCOVE's Modified Dulbeccos Medium with 2 mM Glutamine, 20% fetal calf serum and 1 μg/ml gentamycin at 7.5% CO_2 and 37.5˚C. All cytokine measurements were related to a cell density of $1x10^6$.

Serum samples. Repetitive blood samples were taken from the tail vein of the nude mice (50 μl). When animals were sacrificed at the end of an experiment, blood was collected from the axillary vessels. To collect the serum, blood was centrifuged in Eppendorf tubes for 5 min at 2,000 rpm at room temperature. Serum was analyzed for electrolytes including calcium levels by the Freiburg University Hospital Clinical Chemistry Laboratory using routine procedures.

RNA isolation and gene expression profiling. For each xenograft model, tissues were pooled from 3 different mice and passages, total RNA was isolated from the shock-frozen tumor samples using Qiagen RNAeasy-Kits (Hilden, FRG) according to the manufacturer's instructions. Total RNA was submitted to Affymetrix for labeling, hybridization and bioinformatical evaluation of gene expression profiles. Samples were analyzed in duplicates and gene expression profiles determined by using HG U95Av2 chips. The HG-U95Av2 array represents approximately 10,000 full-length genes, it contains a total of 12,626 oligonucleotide sequences (Affymetrix, Santa Clara, CA, USA, http://www.affymetrix.com/products/arrays/specific/hgu95.affx). Expression data were provided to us as lists of the mean signal intensities of 2 experiments in MS Excel format.

Tumor protein lysates and ELISA. Tumor lysates were prepared in lysis buffer following a published method. The total amount of protein was determined by Bradford assay (10). Cytokine levels such as TNF-α, IL-6 and IL-1 as well as PGE2 were measured by using commercially available ELISA kits from R&D Systems (Minneapolis, USA).

RT-PCR analysis. RT-PCR Analysis were carried out in TE buffer (Qiagen, Hilden, Germany) in a total reaction volume of 50 μl consisting of RNase-free water, Qiagen OneStep RT-PCR buffer, dNTP Mix, Primer 1 (forward), Primer 2 (reverse), OneStep RT-PCR Enzyme Mix (Qiagen) and RNase Inhibitor (Gibco, Invitrogen, Paisley). The total RNA concentration was 2 μg. β-actin was used as an internal standard. The Primer sequences were as follows: For IL-8, Primer 1: 5'-AAGAAACCACCGGAAGG AACC-'3, Primer 2: 5'-ATC-TGGC AACCCTACAACAG-ACC-'3, For IFNgP, Primer 1: 5'- TGTGGAGCAAGGGAC-AAGGC-'3, Primer 2: 5'-GGGTAG AACTGTGGTTCAAGAGG-'3, for DKFZ564I1922, Primer 1: 5'-AATGGCACCCTCCTCACGGTTCG-'3, Primer 2: 5'-GCCTTC AGATGC-GACTTATCCG-'3, for CD27, Primer 1: 5'-TTTCCT TCCTTCCTTCTCGGC-'3, Primer 2: 5'-CTTCTCT-TGTCCTGC CACCACTACC-'3, for PTHrP, Primer 1: 5'-AATAAGTCC-CGAGCGCGAGC-'3, Primer 2: 5'-AGCCTGTT ACCGTGAA TCGAGC-'3, β-actin, Primer 1: 5'-CGTGGACAT CCGCAA AGACC-'3, Primer 2: 5'-TGTGGACTTGGGAGA GGACTGG-'3. The reactions were performed according the following program: 50˚C for 30 min, 95˚C for 15 min; followed by 22 cycles of: 94˚C for 45 sec, 50-60˚C for 45 sec, 72˚C for 90 sec, followed by an additional extension step at 72˚C for 10 min. The PCR products were run on 1.6% agarose gels and stained with ethidium bromide. Signals were visualized under UV-light and photographed.

Immunohistochemistry (IHC). Five μM thick sections were cut from paraffin blocks and tumor tissues were deparaffinized through a series of ethanol and xylenes. Endogenous peroxidase activity was blocked by incubation in 1.5% H_2O_2 in methanol for 30 min at room temperature. For antigen retrieval the sections were treated with citrate buffer and microwaved for 20 minutes. Then, the slides were washed in distilled water and rehydrated in PBS. Immunoreactivity was detected by an immunoperoxidase method (Zymed). Non-specific binding was blocked with 10% goat serum (Sigma, St. Louis, MO, USA) for 60 min. The primary polyclonal anti-PGHS-2 antibody (Oxford Biomedical Research, Oxford, Michigan) against human C-terminus of COX-2 which is absent in COX-1 was added at 4˚C over night. After slides were rinsed 3 times in 200 ml PBS, a biotin labeled secondary anti-rabbit antibody (Zymed) was applied. Slides were washed with PBS, then incubated in streptavidin/peroxidase for 30 min, and rinsed again in PBS. The chromogen diaminobenzidine (Sigma) was added for 5 min. For signal amplification the slides were further incubated with imidazole (1 mg/200 ml PBS, Sigma) and three drops of H_2O_2, and were washed in deionized water; slides were then counterstained with hematoxylin and analyzed by light microscopy (Zeiss Axiovert), photomicrographs were captured with a Kodak digital camera.

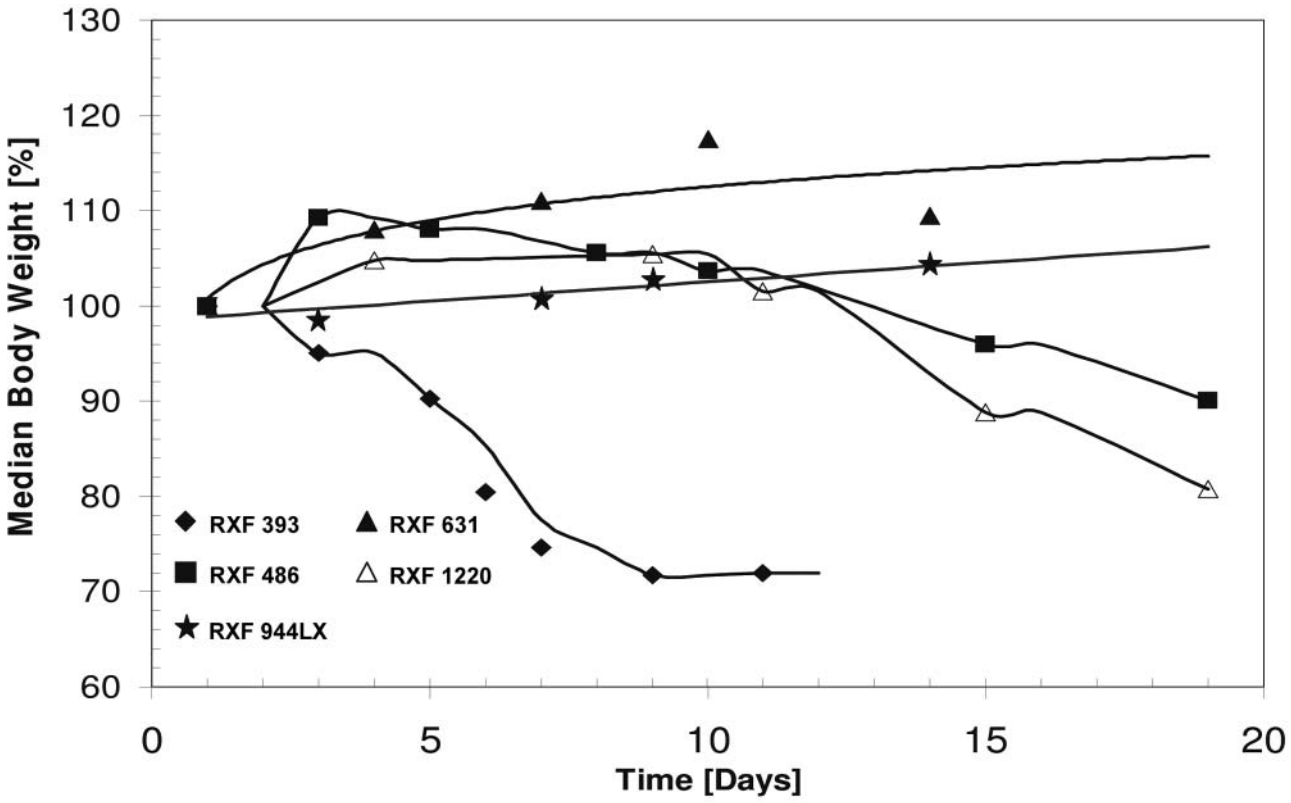

Figure 1. *Body weight change in nude mice with subcutaneously growing renal cell carcinomas. Nude mice bearing the CIX RXF 393 ◆, RXF 486 ■ and RXF 1220 △ show progressive loss of body weight. The renal cell carcinomas RXF 631 ▲ and RXF 944LX ★ do not induce any weight loss in nude mice.*

In vivo treatment with COX-2 inhibitors. Human renal cell tumor xenografts were implanted *s.c.* as fragments (5x5 mm) from tumors in serial passages into both flanks of 6 weeks old nude mice. When tumors reached a size around 50-100 mm^3, animals were randomly allocated into treatment and vehicle control groups (n=5 mice per group). Mice received Vioxx (rofecoxib, MSD, USA) at a dose of 12.5 mg/kg/day or nimesulide (Sigma, Deisenhofen) by oral gavage at a dose of 5 mg/kg/day, daily for up to 3 weeks. Tumor growth and body weight were recorded three times a week. Tumor growth was followed by serial caliper measurement. Tumor volumes were calculated using the formula $(a \times b^2)/2$, where length (a) is the largest dimension and width (b) the smallest dimension perpendicular to the length. Data evaluation was performed by plotting median relative tumor volume against time. Relative tumor volumes were calculated for each single tumor by dividing the tumor volume on day X by the tumor volume on day 0 at the time of randomization. Growth curves were analyzed in terms of tumor inhibition (treated/control, T/C, calculated as median tumor weight of treated divided by median tumor weight of control animals times 100) (12).

Results

Xenograft models of human cancer cachexia. In our studies we used five different human renal cell carcinomas from the Freiburg human tumor xenograft collection (RXF). Three of these, RXF 393, RXF 486 and RXF 1220 induce a characteristic cachectic syndrome (CIX) after implantation into nude mice, whereas the other two, RXF 944LX and RXF 631, do not induce cachexia (non-CIX). The extent of body weight loss caused by the xenotransplants varied: RXF 393 induced the most pronounced body weight loss, namely 20% after only 6 days, whereas RXF 1220 and RXF 486 caused a 20% loss of body weight at 18 and 21 days after transplantation respectively (Figure 1). In contrast, RXF 944XL and RXF 631 bearing nude mice showed a moderate increase of body weight, this was related in part to the increasing tumor burden.

Identification of new cachexia-associated genes by Affymetrix profiling. To obtain a more complete insight into the molecular pathology of cancer cachexia, we performed genomic profiling analyses of CIX *versus* non-CIX in order to identify genes, which are associated with the paraneoplastic syndrome. We used the full-length human Affymetrix GeneChip expression arrays and compared mean signal intensities among CIX and the non-CIX models (Figure 2). We ranked all 12,200 genes from highest to lowest expression for each xenograft and generated a list of commonly up-regulated genes for the CIX. Next, we

Table I. *Novel distinct cachexia-associated genes from Affymetrix profiling.*

Novel cachexia-associated genes	Ratio CIX *vs*. non-CIX	Average RXF CIX	Average RXF non-CIX
Cadherin-6	91.0	1375.2	15.1
Human cyclooxygenase-2 (COX-2)	76.0	539.6	7.1
Human parathyroid hormone related peptide (PTHrP)	50.6	1998.6	39.5
Human interferon-γ treatment inducible protein (IFNgP)	29.3	3544.4	121.2
Human interleukin 8 (IL-8)	20.0	4817.7	240.8
DKFZ564I1922	16.7	8430.6	503.4
Human CD-27 (TNF-α ligand superfamily protein)	12.3	3335.9	271.3
CD-13	5.5	3264.4	594.6
Human adipophilin	2.7	6184.9	2255.8
TGF-β superfamily protein	2.1	1410.4	670.4

Ratio = average signal intensity of CIX on the Affymetrix chip *versus* signal intensity of non-CIX.

extracted the expression levels of the same genes from the non-CIX and generated a CIX:non-CIX ratio (Table I). For the validation of the array data we analyzed the expression of selected genes by semi-quantitative RT-PCR analysis. Among the most highly expressed genes were several cytokine-related and novel proteins. As shown in Table I we could identify cadherin-6, the human parathyroid hormone related peptide (PTHrP), and COX-2 with a very high CIX:non-CIX ratio (Table I, Figure 2). The expression of the proinflammatory cytokine-associated genes interleukin-8, interferon-γ treatment inducible protein (IFNgP), DKFZ564I1922 and TNF-α ligand superfamily protein (CD-27) were also remarkable. We only listed those genes in Table I, which have previously not been associated with cancer cachexia and for which we performed confirmatory RT-PCR analyses (Figure 3A); highly expressed genes with known function are not shown and were not examined in more detail.

RT-PCR of new cachexia-associated genes. RT-PCR was performed to confirm the differential expression of novel genes in CIX as identified by Affymetrix array (Table I). Consistent with the expression array data, IL-8 was detectable in RXF 393 and RXF 486, but not in RXF 631 and only weakly in RXF 944LX. Similarly, IFNgP, DKFZ564I1922, TNF-α ligand superfamily protein (CD-27), and parathyroid hormone related peptide (PTHrP) were strongly expressed in CIX, but not or minimally in non-CIX (Figure 3A). Moreover, in the case of PTHrP the extend of gene expression was associated with the extend of cachexia development in tumor-bearing nude mice (Figure 1). With 3,440 units, RXF 393 showed the highest signal intensity followed by RXF 486 with 554 units. The non-CIX RXF 631 and RXF 944 expressed only very minimal levels of PTHrP (Figure 3 A and B). Moreover, the expression profile of PTHrP mRNA was associated with remarkably elevated calcium serum levels in CIX mice. RXF 393-bearing nude mice showed the highest value of calcium with 4,7 mmol/L. RXF 486 had also elevated calcium levels compared to tumor-free control animals and non-CIX (Figure 3C).

Pro-inflammatory cytokines and cachexia. TNF-α, IL-1 and IL-6 protein levels were detected by enzyme-linked immunosorbent assay in culture supernatants of isolated primary cells from xenografts grown in nude mice. As shown in Figure 3D all of the examined cachexia-inducing xenografts had elevated amounts of TNF-α, whereas the non-CIX did not produce the cytokine. RXF 393 showed the highest TNF-α levels with 220 pg per 10^6 cells followed by the xenografts RXF 1220 and RXF 486 with 105 pg and 50 pg per 10^6 cells, respectively. The extent of TNF-α secretion was clearly associated with the severity of cachexia development. As described above, RXF 393 caused the most pronounced cachexia and produced the highest TNF-α value (Figure 3D). IL-1 was only produced by cells derived from the xenografts RXF 393 and RXF 486. Primary cultures of RXF 1220 and the non-CIX RXF 944LX and RXF 631 did not produce any IL-1 (Figure 3D). These results were confirmed in independent experiments with culture supernatant of permanent cell lines as well as with tumor tissues and showed the same pattern of cytokine expression. In normal human kidney tissue, no cytokines were detectable (data not shown). In contrast, high levels of IL-6 were measurable in all xenograft tissues and in the serum of tumor-bearing nude mice, independent of the extent of cachexia induction.

Cyclooxygenase-2, prostaglandin E2 and cachexia. Because COX-2 and its product prostaglandin E2 (PGE2) are key down-stream effectors of cytokine activity, we compared their expression levels in CIX and non-CIX. Immunohistochemical analyses revealed an elevated expression of COX-2 in CIX, whereas non-CIX showed only a moderate expression of COX-2 (Figure 4A-E). Accordingly, the CIXs RXF 393,

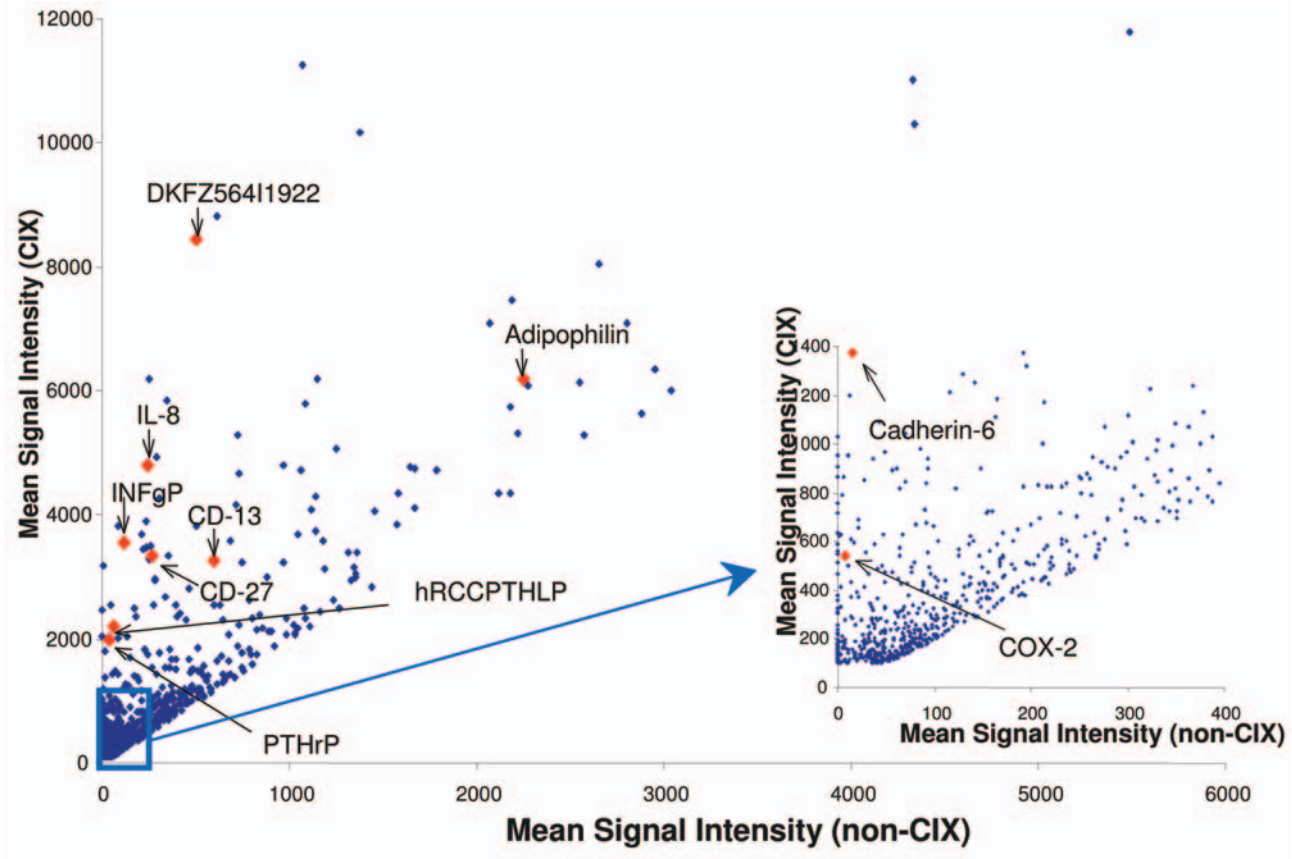

Figure 2. *Scatter plot comparing gene expression of CIX and non-CIX by HG-U95Av2 Affymetrix DNA array. Only genes with a mean signal intensity of higher than 100 fluorescence units and a CIX to non-CIX ratio of >3 were included in the analysis. The HG-U95Av2 array contains a total of 12,200 human cDNAs of full length genes. The insert is a magnification of genes that were differentially expressed in CIX and non-CIX but at a lower level. hRCCPTHLP, human renal cell cancer parathyroid hormone-like peptide.*

RXF 486 and RXF 1220 express high levels of PGE2 with up to 979 pg per 10^6 cells measured in RXF 486 (Figure 5A-B), whilst in non-CIX, only very small levels of PGE2 were found. Moreover, paralleling the expression pattern of PGE2, TNF-α synthesis was elevated (Figure 5C). These data suggest the involvement of COX-2 in the development of tumor cachexia. To test whether this observation would translate into a delay of the onset of cachexia in CIX bearing animals when COX-2 is inhibited, we used the specific COX-2 inhibitors Vioxx (rofecoxib) and nimesulide. The treatment of CIX-inducing RXF 486 cells with COX-2 inhibitors led to a marked reduction of PGE2 synthesis in cell culture supernatants (Figure 5A and B). Furthermore, a reduction of TNF-α in lysates from the CIXs RXF 393 and RXF 486 was seen after nimesulide and Vioxx (only tested in RXF 393) treatment (Figure 5C).

In vivo administration of COX-2 inhibitors by daily oral gavage in CIX-bearing nude mice, confirmed the central role of COX-2 in the development of cachexia by causing a stabilization of body weight while tumor growth was not inhibited. As shown in Figure 5D for RXF 393 xenografts, the body weight remained nearly constant for the duration of the treatment with COX-2 inhibitors (11 days). Nimesulide however was more effective than Vioxx (Figure 5C). Control vehicle treated animals had lost 30% of their body weight after 10 days, while Vioxx treated mice were at 75% of their original body weight after 15 days. Nimesulide treated mice showed a body weight loss of only 20% after 25 days. Similar results were observed in RXF 486 bearing nude mice (data not shown). In control experiments with the non-CIX RXF 631, body weight and tumor growth remained unaffected.

Discussion

Cachexia is a progressive wasting syndrome characterized by extensive loss of adipose tissue and skeletal muscle. It occurs in about half of all cancer patients. The presently available data clearly indicate that tumor cachexia has multifunctional causes (4). Various mediators such as

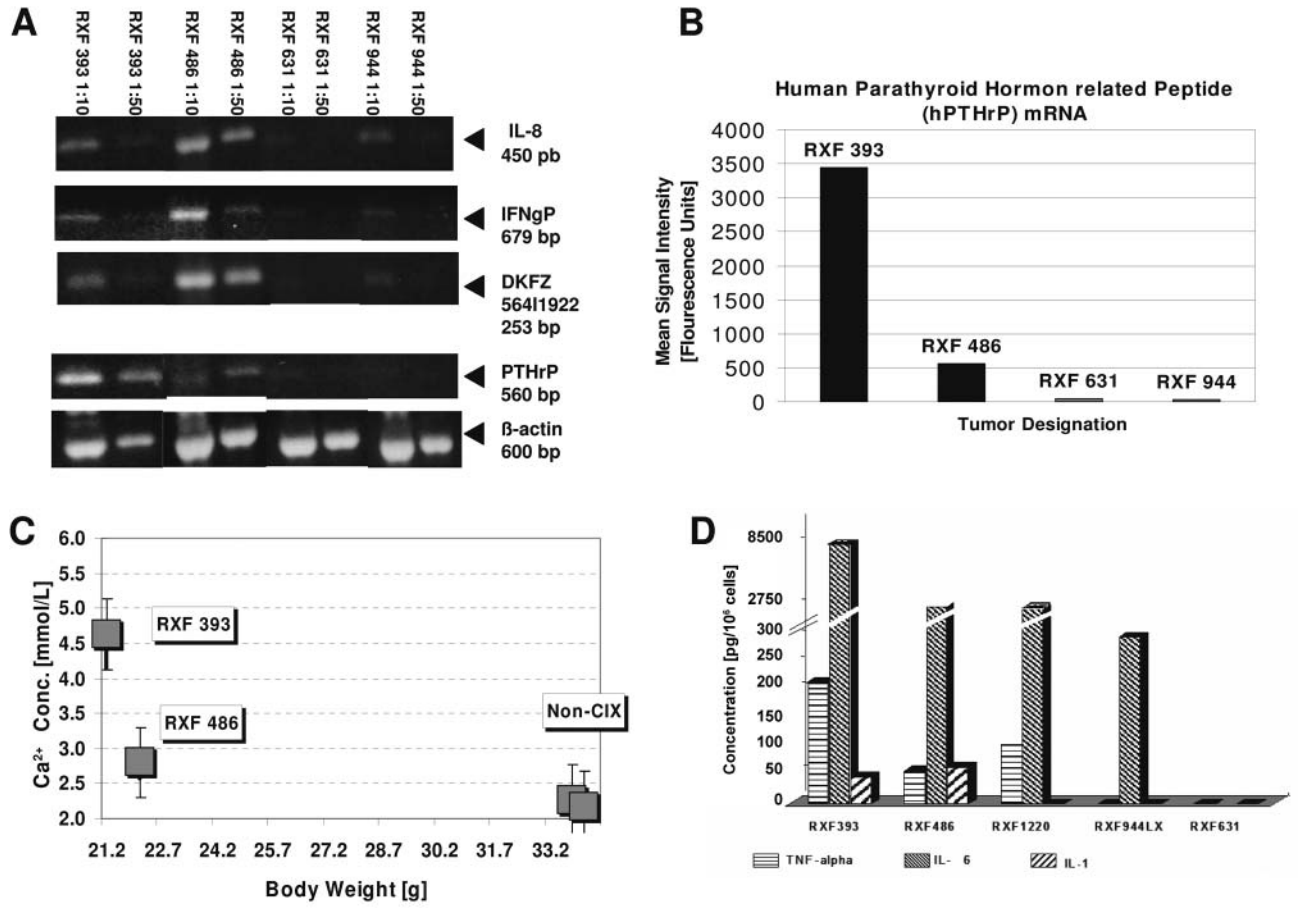

Figure 3. *A) Semi-quantitative RT-PCR analyses of IL-8, IFNgP, DKFZ 564I1922 and PTHrP mRNA in CIX RXF 393 and RXF 486 and non-CIX RXF 631 and RXF 944 LX confirm the results from the Affymetrix array (see Figure 2). CIX show higher mRNA levels for all genes compared to non-CIX. β-actin was used as a housekeeping gene. 1:10/1:50 is the dilution factor of RNA before RT-PCR. B) mRNA expression levels of PTHrP as determined by Affymetrix array in the CIX RXF 393 and RXF 486, and the non-CIX RXF 631 and RXF 944 LX. C) Serum Ca^{2+} concentrations in nude mice bearing renal cell carcinomas. Nude mice bearing the CIX RXF 393 and RXF 486 show elevated serum calcium concentrations, whereas non-CIX do not. Serum Ca^{2+} levels mirror PTHrP concentrations in the cachexia inducing renal cell carcinomas RXF 393 and RXF 486. D) Comparison of TNF-α, IL-6 and IL-1 protein concentrations in primary culture supernatants of the CIX RXF 393, RXF 486 and RXF 1220 and the non-CIX RXF 944LX and RXF 631 (the IL-6 concentration in RXF 631 culture supernatants was not measured).*

pro-inflammatory cytokines, neuroendocrine hormones and tumor specific factors have been implicated.

Pro-inflammatory cytokines, TNF-α, IL-1, IL-6, interferon-γ, prostaglandins and the tumor-specific factor PIF (proteolysis inducing factor) have been studied in more detail, but the mechanism of action of these factors remains still poorly understood (3-4).

The aim of our study was therefore to gain greater insight into the molecular pathogenesis of cachexia. Because the known cachectic factors can not sufficiently describe cachexia symptoms, we explored the expression of cachexia-associated genes by DNA-array profiling and confirmed their cachexia-specific expression by semi-quantitative RT-PCR analyses. Five human renal cell carcinomas were available for these studies from the Freiburg human tumor xenograft collection (XF); three of which cause cachexia in nude mice, two do not. Additionally, we used permanent cell lines that have been established from the xenografts, and when injected into nude mice reflect the CIX and non-CIX phenotype. The availability of these model systems provided us with optimal conditions for the identification of cachexia-inducing factors. Thus, we were able to confirm that TNF-α, IL-1 and IL-6 are important mediators in the development of cancer cachexia, further validating our model systems. In the cachexia-inducing renal cell cancers, a clear correlation exists between TNF-α expression and cachexia development, more importantly however, we have established a causative relationship between COX-2 expression and cachexia. First,

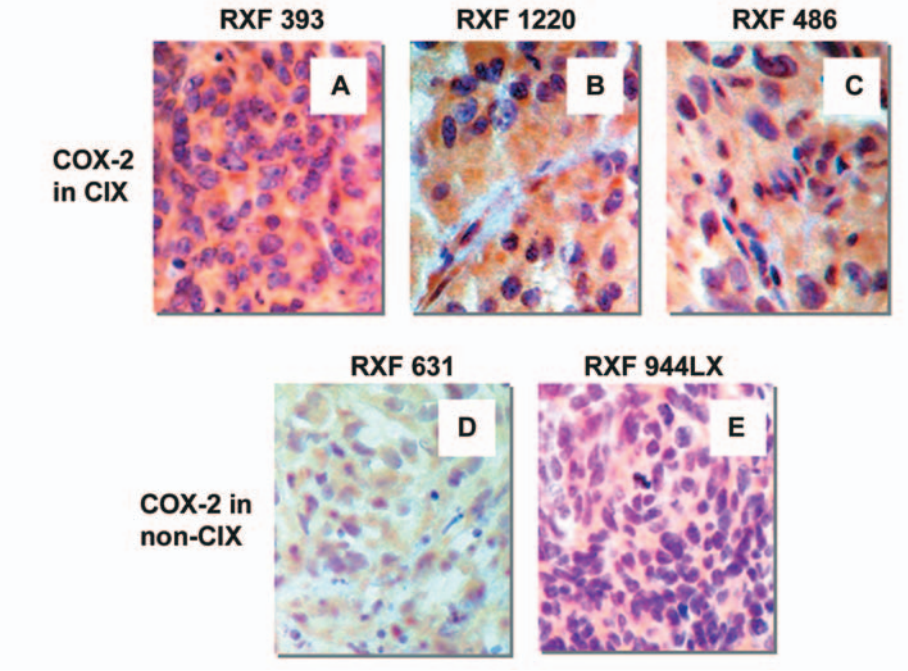

Figure 4. *Cyclooxygenase 2 (COX-2) expression in renal cell carcinomas. Cachexia-inducing renal cell carcinomas RXF 393 (A), RXF 1220 (B) and RXF 486 (C) show high expression of COX-2 in the cytoplasm, whereas non-cachexia inducing renal cell carcinoma tissues RXF 631 (D) and RXF 944LX (E) do not show elevated COX-2 levels.*

we showed that COX-2 expression is distinctly elevated simultaneously with an increase in PGE2 and TNF-α synthesis. Second, by blocking PGE2 synthesis with COX-2 inhibitors, we could show a drastic slow down of body weight loss in CIX-bearing nude mice and this effect translated into a reduction of TNF-α expression. Control mice lost 30% of their body weight after 10 days, whereas Vioxx treated animals were still at 75% of their body weight after 15 days and nimesulide treated mice had lost only 20% of their body weight after 25 days. Most importantly, the COX-2 inhibitory agents had no effects on tumor growth. These data were reproduced in 3 independent experiments.

Our results are very similar to those published by Hussey and Tisdale with the COX-2 inhibitor meloxicam in the mouse MAC16 tumor model (7). But in contrast to their results, in our study, the growth of CIX in nude mice was not influenced by COX-2 inhibition. Together our *in vitro* and *in vivo* data clearly implicate TNF-α and other pro-inflammatory mediators as a cause of the development of cancer cachexia.

Additional factors that were identified by us in CIX, but not non-CIX renal cell carcinomas include a pronounced increase in serum calcium concentrations. The CIX also produce high level of PTHrP whereas in non-CIX, no PTHrP or elevated calcium concentrations could be found. It is believed that hypercalcemia is due to the release of certain mediators by cancer cells. One of the latter is PTHrP, other potential mediators are TNF-α, IL-1 and IL-6 (14-15). Thus, suggesting that cachexia is linked to the appearance of hypercalcemia. Recent reports indicate that PTHrP itself is a pro-inflammatory factor that is able to induce the cachectic cytokine network (16-17). In therapeutic studies using an anti-PTHrP antibody, the extent of cachexia was significantly attenuated in LC-6 lung tumor bearing nude mice (18).

To gain further insight into the regulatory molecular networks that govern cancer cachexia, we employed RNA-expression profiling by using the Affymetrix DNA-chip technology followed by semi-quantitative RT-PCR analysis. Besides the already described inflammatory factors, we discovered several new genes that are associated with inflammatory events and may represent new cachexia factors that could be useful as molecular targets for drugs or as disease markers. In particular, we identified three new genes which were highly expressed in CIX, but not non-CIX, they include IL-8, TNF-α ligand superfamily protein, IFNgP, and DKFZ569I1922. The identification of IL-8 as a cachectic factor is not surprising, because its synthesis is stimulated by TNF-α and other cytokines.

TNF-α ligand superfamily protein is a protein with homologies to TNF. It belongs to a new family of cytokines and plays a role in induction of apoptosis (19-21).

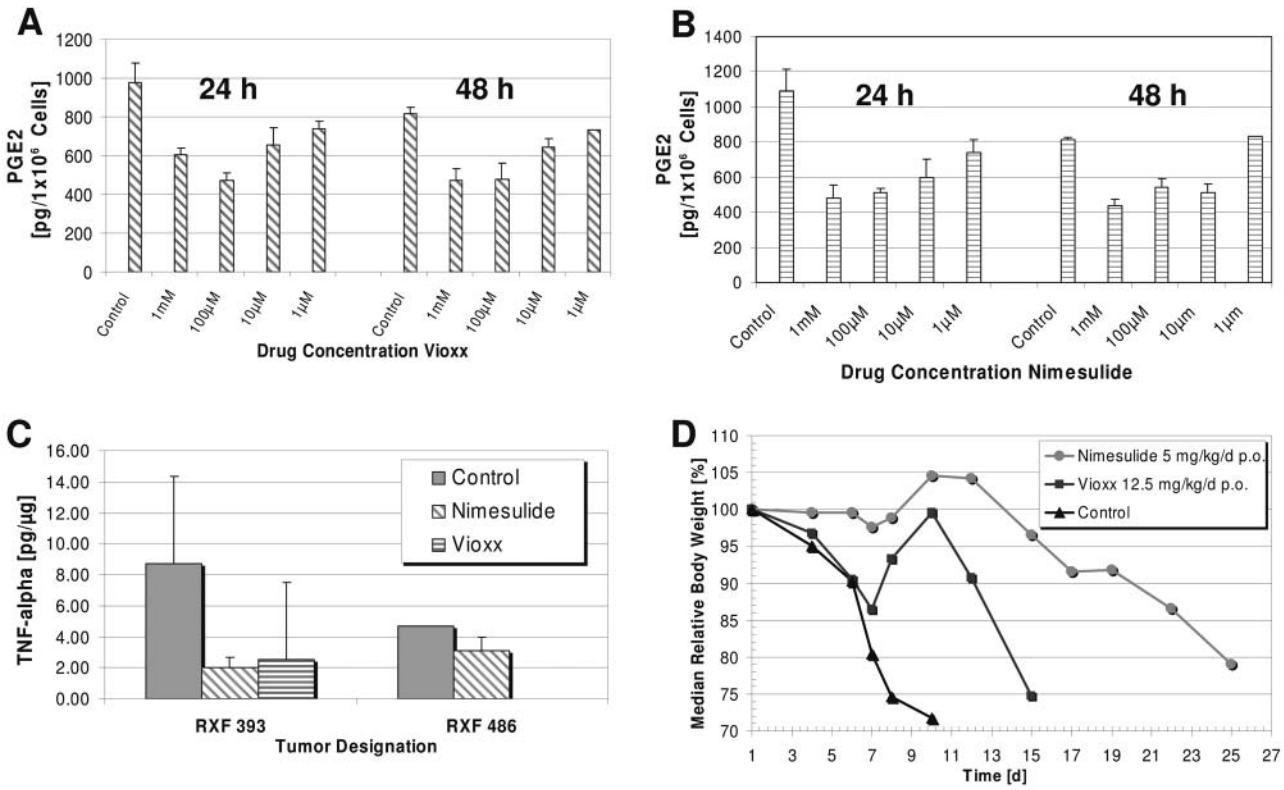

Figure 5. *Protein concentrations of PGE2 in cell culture medium of the CIX cell line RXF 486 after in vitro treatment with (A) Vioxx (rofecoxib) and (B) nimesulide. The treatment with the specific COX-2 inhibitors nimesulide and rofecoxib for 24h and 48h induces a concentration dependent suppression of PGE2 synthesis. C. TNF-α concentrations in whole cell lysates from tumor tissue of the CIX RXF 393 and RXF 486 after treatment of nude mice with COX-2 inhibitors. The TNF-α concentrations in tumor tissues were markedly reduced after chronic, oral administration of the COX-2 inhibitors rofecoxib (12.5 mg/kg/d) and nimesulide (5mg/kg/d). D. Relative change in body weight of nude mice bearing the cachexia-inducing renal cell carcinoma RXF 393 under treatment with COX-2 inhibitors as in C. When treatment was terminated on day 11, control mice had lost >30% of their body weight. Animals treated with Vioxx had lost 25% body weight after 15 days. In contrast, nimesulide treated mice showed a body weight loss of only 20% after 25 days. Data are representative of 3 independent experiments. ▲ Control, ● nimesulide 5 mg/kg/d ■ Vioxx/rofecoxib 12.5 mg/kg/d.*

Interferon-γ treatment inducible protein is a pro-inflammatory peptide which is induced by interferon-γ. The fact that IFN-γ is considered a cachectic factor and together with TNF-α may be responsible for the lowering of muscle protein syntheses, this peptide could be another cachexia-associated cytokine (22-25).

Little is known about the function of DKFZ564I1922. However, it has been detected in the cartilage of patients with arthritis (26). Because arthritis is a severe inflammatory disease, DKFZ564I1922 might be a cachexia-mediating cytokine.

The results described in this study, demonstrate that the development of experimental cachexia by human renal tumor xenograft models is more complex than previously described in reports on the role of pro-inflammatory cytokines such as TNF-α, IL-1, or IL-6 (27). Our experiments demonstrate that the cachectic syndrome represents a multifactorial, severe inflammatory event in which a number of different mediators play a role. Immunological parameters appear useful in assessing the conditions of the disease as well as for a therapeutic intervention. An effective treatment of cancer cachexia will however require complex combinations of drugs that target pro-inflammatory cytokine networks.

While our renal cell cancer xenograft models were very homogenous in their gene and protein expression profiles, it is possible that other tumor histologies show a different molecular fingerprint of cachexia mediating factors. Our studies should thus be extended to and validated with clinical tissue specimens. If our findings are confirmed, cachexia-associated gene profiles could be used for early detection of the syndrome in individual patients, and COX-2 inhibitors together with therapeutic antibodies against pro-inflammatory cytokines could be administered to intercept cancer cachexia development in its early stages.

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References

1 Andreyev HJN, Norman AR, Oates J and Cunningham D: Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignacies? Eur J Cancer *34*: 503-509, 1998.
2 Fearon KC and Moses AG: Cancer cachexia. Int J Cardiol *85*: 73-81, 2002.
3 Tisdale MJ: Pathogenesis of cancer cachexia. J Support Oncol *1*: 159-168, 2003.
4 Muscaritoli M, Bossola M, Aversa Z, Bellantone R and Fanelli FR: Prevention and treatment of cancer cachexia: new insights into an old problem. Eur J Cancer *42*: 31-41 2006.
5 Dunlop RJ and Campbell CW: Cytokines and advanced cancer. J Pain Symptom Manag *20*: 214-232, 2000.
6 Tisdale MJ: Biology of cachexia. J Natl Cancer Inst *89(23)*: 1763-1773, 1997.
7 Hussey HJ and Tisdale MJ: Effect of the specific cyclooxygenase-2 inhibitor meloxicam on tumor growth and cachexia in a murine model. Int J Cancer *87*: 95-100, 2000.
8 Gelin J, Andersson C and Lundholm K: Effects of indomethacin, cytokines, and cyclosporin A on tumor growth and the subsequent development of cancer cachexia. Cancer Res *51*: 880-885, 1991.
9 Mulligan HD, Mahony SM, Ross JA and Tisdale MJ: Weight loss in a murine cachexia model is not associated with the cytokines tumour necrosis factor-alpha or interleukin-6. Cancer Lett *65*: 239-243, 1992.
10 Burger AM, Leyland-Jones B, Banerjee K, Spyropoulos DD and Seth AK: Essential roles of IGFBP-3 and IGFBP-rP1 in breast cancer. Eur J Cancer *41*: 1515-1527, 2005.
11 Workman P, Twentyman P, Balkwill F, Balmain A, Chaplin D, Double J, Embleton J, Newell D, Raymond R, Stables J, Stephens T and Wallace J: United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). Br J Cancer *77*: 1-10, 1998.
12 Fiebig HH, Berger DP, Dengler WA, Wallbrecher E and Winterhalter BR: *In*: Immunodeficient Mice in Oncology. Fiebig HH, Berger DP (eds.). Contrib Oncol Basel Karger Verlag, pp. 321-351, 1992.
13 Burger AM: Standard TRAP assay. *In*: Telomeres and Telomerase: Methods in Molecular Biology. Double JA, Thompson MJ (eds.). Vol. 191, chap. 7, The Humana Press Inc, Totowa, NJ-USA, pp. 109-124, 2002.
14 Cahlin C, Körner A, Axelsson H, Wang W, Lundholm K and Svanberg E: Experimental cancer cachexia: the role of host-derived cytokines interleukin (IL-6), IL-12, interferon γ, and tumor necrosis factor α evaluated in gene knockout, tumor-bearing mice on C57 B1 background and eicosanoid-dependent cachexia. Cancer Research *60*: 5488-5493, 2000.
15 Dodwell DJ: Malignant bone resorption: cellular and biochemical mechanisms. Ann Oncol *3*: 257-267, 1992.
16 Martin-Ventura JL, Ortego M, Esbrit P Hernandes-Presa MA, Ortega L and Egido J: Possible role of parathyroid hormone-related protein as a proinflammatory cytokine in atherosclerosis. Stroke *34*: 1783-1789, 2003.
17 Ogata E: Parathyroid hormone-related protein as a potential target of cancer-associated morbidity. Cancer *88*: 2909-2911, 2000.
18 Sato K, Onuma E, Yocum RC and Ogata E: Treatment of malignancy-associated hypercalcemia and cachexia with humanized anti-parathyroid hormon-related protein antibody. Seminars in Oncology *30*: 167-173, 2003.
19 Lewis MI: Apoptosis as a potential mechanism of mucle cachexia in chronic obdtructive pulmonary disease. Am J Crit Care Med *166*: 434-436, 2002.
20 Sharma R and Anker SD: Cytokines, apoptosis and cachexia: the potential for TNF antagonism. Int J Cardiol *85*: 161-171, 2002.
21 UniGene Cluster Hs.99899
22 Moser B, Loetscher M, Piali L and Loetscher P: Lymphocyte responses to chemokines. Int Rev Immunol *16*: 323-344, 1998.
23 Tisdale MJ: Protein loss in cancer cachexia. Science 289, 2000.
24 Moser B, Loetscher M, Piali L and Loetscher P: Lymphocyte responses to chemokines. Int Rev Immunol *16*: 323-344, 1998.
25 Salomon I, Netzer N, Wildbaum G, Schif-Zuck S, Mao G and Karin N: Targeting the funktion of IFN-gamma-inducible protein 10 suppresses ongoing adjuvant arthritis. J Immunol *169*: 2685-2693, 2002.
26 Crowl RM and Luk D: Identification of the gene encoding Adlican, a novel protein expressed in human arthritic tissues (http://www.ebi.uniprot.org/entry/Q9NR99).
27 Argiles JM and Lopez-Soriano FJ: The role of cytokines in cancer cachexia. Med Res Rev *19*: 223-248, 1999.

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