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Partial regression of conventional renal cell carcinoma displays markers of wound repair

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ABSTRACT

Aims During detailed analysis of H&E-stained histological slides of 710 unbiased conventional renal cell carcinomas (cRCCs), 141 tumours displayed partial regressive changes showing strong similarity to that of wound healing. We aimed to analyse the molecular processes occurring in regressive tumours.

Methods Immunohistochemistry was applied to analyse the signalling molecules in 12 selected tumours, and statistical analysis was used to estimate the correlation between regression and the outcome of the disease.

Results The regressive areas displayed inflammatory granulation tissue expressing transforming growth factor beta-1 (TGFB1), interleukin-1 beta and interleukin-6 (IL1B and IL6), proliferation of alpha smooth muscle actin (αSMA) positive naïve activated fibroblasts and a diffuse fibronectin 1 (FN1) network. In the central areas of regressive tissues, parallel-running myofibroblasts showed FN1, collagen type I alpha 1 (COL1A1) and collagen type III alpha 1 (COL3A1) positive immunoreaction. Partial tumour regression is associated with a better postoperative course of the disease.

Conclusions Partial regression is a frequent event in cRCCs. Recognising complex molecular processes involved in tumour regression might help to find a way towards 'healing' cRCC.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Nearly 100 case reports of spontaneous regression of primary and metastatic renal cell carcinoma have been published since 1900.

WHAT THIS STUDY ADDS

⇒ Histological analysis of 710 renal cell carcinomas discovered spontaneous regression in 20% of tumours. Immunohistochemistry of signalling molecules revealed parallels of spontaneous regression to the wound healing process.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our study highlighted the need for further studies to understand the molecular mechanism of partial regression to achieve clinically relevant interventions.

INTRODUCTION

Spontaneous regression is defined as remission or disappearance of a cancer in the absence of adequate treatment, which can be demonstrated by microscopic examination. The remission may be partial or complete. Several well-documented cases of spontaneous regression of distinct types of cancers have been reported, most frequently in patients with lymphoma, skin cancer and melanoma.[1] Nearly all cases of spontaneous remission are associated with acute infection and high fever. Best known is Coley's toxin which induces high fever and prolongs the survival of patients having sarcomas, lymphomas and melanomas longer than 5 years.[2] Activation of the immune system plays an important role in tumour regression.[3 4] Stimulation of the immune system might be responsible for the efficient treatment of superficial bladder cancer with BCG.[3] Intravesical application of BCG results in increased production of interleukins (IL) such as IL6, IL12 among others.[5]

Conventional renal cell carcinoma (cRCC) is an aggressive tumour, nearly 40% of the patients have a metastatic disease at the time of operation or will develop during the postoperative course.[6] An estimated 1% of RCCs, even metastatic ones may undergo spontaneous regression.[7] From 1900 to 1965, 31 well-documented case reports on spontaneous regression of RCCs have been published.[8] Paying more attention to this biological phenomenon, 68 case reports were published between 1966 and 1987.[9] Although complete regression of cRCC is rare, partial regression occurs frequently without reporting in histological records.

The aim of this study was to analyse partial regressive changes in cRCC with distinct cellular morphology, tumour grading and estimate its correlation to postoperative outcome of the disease. Immunohistochemistry was applied to analyse the molecular changes in regressive areas resembling wound healing.

MATERIALS AND METHODS

Patients and tissue samples

Formalin-fixed, paraffin-embedded cRCC samples obtained from 710 patients operated consecutively between 2000 and 2015 at the Department of Urology, Medical School, University of Pecs, Hungary were included in this study. In this cohort, 61 patients had metastasis at the time of surgery and 112 patients showed progression during the postoperative course. Preoperative clinical staging included CT scans of the abdomen and chest. Patients were controlled every 6 months by abdominal ultrasound and measurement of serum creatinine and eGFR (estimated glomerulus filtration rate), and annually by CT. The male/female ratio was 416 (59%) to 294 (41%), the mean age was 57±11 years (20–87) years and the mean size of tumours was 49±24 mm. The median postoperative

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follow-up was 67 months (range 34–114 months). Follow-up was designed as a time from surgery until the last recorded control, or cancer-specific death. Patients who died from disease other than renal cancer were excluded from the study. Data on regular follow-up and tumour-specific death were obtained from the Tumour Registry of the Department of Urology in accordance with relevant institutional guidelines and regulations. Histological diagnosis of cRCC was confirmed by an experienced pathologist (GK) by applying the Heidelberg Classification.[10] According to this classification approximately 70%–80% of conventional RCCs are composed of 'clear' cells, the rest of mixed clear-eosinophilic or pure 'eosinophilic' cells or showed rhabdoid or sarcomatous histology.[11] We used the Heidelberg Classification because it is based on roboust tumour-specific chromosomal/DNA alterations. For tumour staging the 2016 TNM system[12] and for grading a three-tiered grading system[11] based on alterations of tumour cell nuclei were used.

Immunohistochemistry

12 tumours with characteristic regressive histology were selected for immunohistochemistry. After dewaxing and rehydration of slides, heat-induced epitope retrieval was performed in 10 mM sodium citrate buffer, pH 6,0 or EnVision FLEX Target Retrieval Solution, high pH (DAKO, Glostrup, Denmark) in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, The Netherlands). Endogenous peroxidase activity and unspecific binding sites were blocked with Envision FLEX Peroxydase Blocking Reagent (DAKO) for 10 min at room temperature. Slides were incubated for 60 min at room temperature with anti-IL6 polyclonal rabbit antibody (ab PAI-26811, Thermo Fisher Budapest, Hungary) at the dilution of 200, anti-IL1B monoclonal mouse antibody (AM06692SU-N, Origene Rockville, Maryland, USA) at the dilution of 1:200, anti-TGFB1 (transforming growth factor beta-1) polyclonal rabbit antibody (SAB4502954, Sigma Aldrich, Budapest, Hungary) at 1:100 dilution, anti-alpha smooth muscle antigen (αSMA) monoclonal rabbit antibody (ab124964 Abcam) at 1:1000 dilution, anti-fibroblast-associated protein alpha (FAPα) polyclonal rabbit antibody (ab207178, Abcam) at dilution 1:200, anti-FN1 (fibronectin 1) monoclonal rabbit antibody (ab 32419, Abcam) at 1:250 dilution, anti-COL1A1 (collagen type I alpha 1) polyclonal rabbit antibody (PA5-29569, Thermo Fisher Budapest, Hungary) at the dilution of 1:500, anti-COL3A1 (collagen type III alpha 1) polyclonal rabbit antibody (PA1-28870, Thermo Fisher Budapest, Hungary) at 1:200 dilution, anti-MMP9 polyoclonal rabbit antibody (AV33090, Sigma-Aldrich) at 1:200 dilution. FLEX horseradish-peroxidase conjugated secondary antibody (DAKO) was applied for 20 min at room temperature. The signal was visualised with amino-ethyl-carbazol (AEC) or 3,3′-diaminobenzidin (DAB) (DAKO). Tissue sections were counterstained with Mayer's haematoxylin (Lillie's modification, DAKO) and after 10 s bluing in ammonium-hydroxide solution, were mounted by Glycergel (DAKO). For negative control, the primary antibody was omitted. Photographs were taken by a Leitz DMRBE microscope, equipped with HC PLAN APO 20×0.70 objective, and a ProgRes C14 camera (Leitz, Wetzlar, Germany).

Statistical analysis

Data analysis was performed with the SPSS Statistics software package V.20.0 (IBM, 35 Armonk, New York, USA). The effect of regression on the tumour progression was estimated with Kaplan-Meier analysis, and the comparison of survival curves was made with the Log rank test. Patients alive and disease-free during the follow-up period were censored. Differences were considered significant at $p<0.05$.

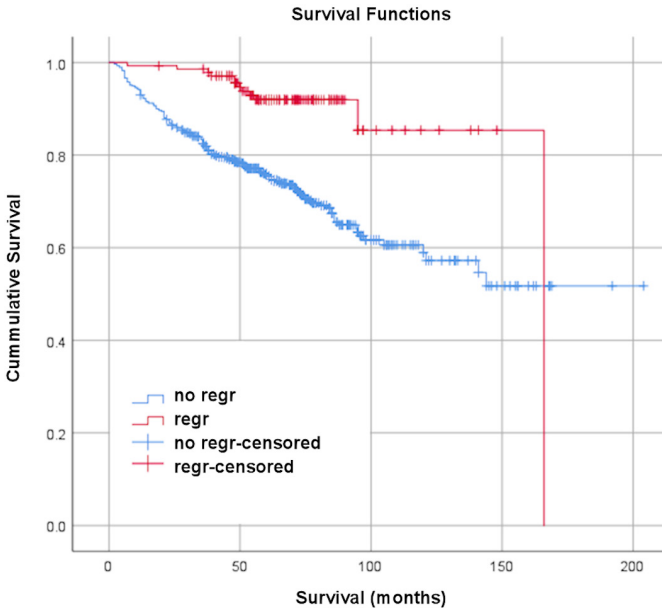

Figure 1 Kaplan-Meier analysis. Significant correlation between regressive alterations and tumour progression (p<0.001).

RESULTS

Regressive histology and tumour progression

During the detailed analysis of H&E-stained histological slides of 710 unbiased cRCCs for cellular heterogeneity and new nuclear grading,[11] we recognised 141 tumours with partial regressive changes. The regressive histology was associated with tumour-free survival of patients (figure 1). Cox regression analysis showed that 92.8% of patients with cRCCs showing regressive changes, whereas 75.7% of patients without regressive tumour alterations were free of disease at the end of 5 years follow-up period (p<0.001). Estimated survival of patients with regressive tumours was 151 (140–162)±6 months and without regressive alterations 136.0 (126–145)±5 months by overall survival of 140 (130–150)±5 months. Multivariate analysis showed that regressive alteration was not an independent prognostic factor.

Table 1 Distribution of grade and regression in tumours with distinct cellular characteristics

		Cellular characteristics (% in parenthesis)					
	Total 710	Clear 460 (65)	Clear/eos 110 (16)	eos 109 (15)	rhabd 23 (3)	sarc 8 (1)	P value
Grade							p<0.001
G1	442 (62)	411 (89)	27 (25)	4 (4)	0	0	
G2	170 (24)	47 (10)	65 (59)	58 (53)	0	0	
G3	98 (14)	2 (<1.0)	18 (16)	47 (43)	23 (100)	8 (100)	
Regression							p<0.001
No	569 (80)	340 (74)	95 (86)	104 (95)	22 (96)	8 (100)	
Yes	141 (20)	120 (26)	15 (14)	5 (5)	1 (4)	0	
Progression							p<0.001
No	532 (75)	410 (89)	68 (62)	48 (44)	3 (13)	3 (37)	
Yes	178 (25)	50 (11)	42 (38)	61 (56)	20 (87)	5 (63)	

clear, pure clear cell; clear/eos, mixed clear and eosinophilic cells; eos, pure eosinophilic cells; RCC, renal cell carcinoma; rhabd, rhabdoid cells; sarc, sarcomatous RCC.

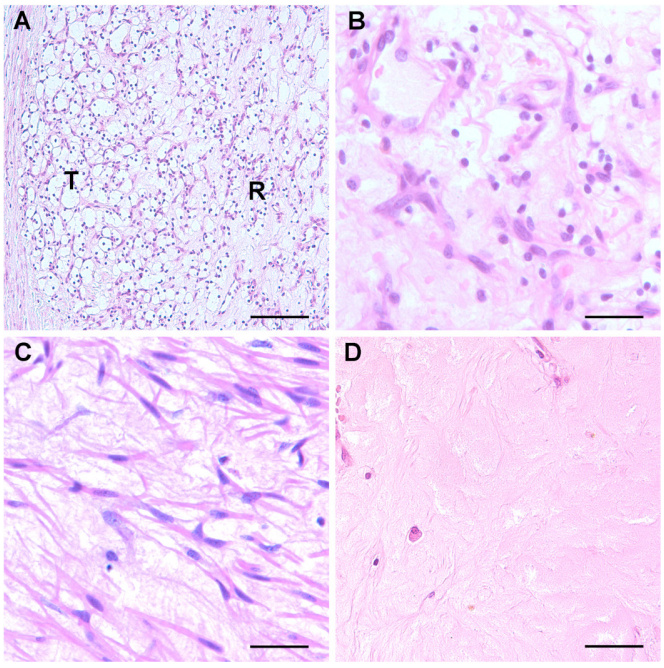

Figure 2 Histology of regressive alterations. (A) Trabecular growing cRCC (T) under the capsule and tumour cell-free regressive granulation (R). (B) Granulation tissue displaying strong proliferaion of endothelial (myo-endothelial) cells and high number of immune cells. (C) Proliferation of myofibroblasts with characteristic stellate and spindle morphology and eosinophilic cytoplasm. (D) Central homogeneous fibrotic area displaying only few cellular components. H&E staining. Scale bars=100 µm (A), 40 µm (B–D). cRCC, conventional renal cell carcinoma.

Regression, grade and cytomorphology

The occurrence of tumour grade, regression and progression within each cytomorphological group is shown in table 1. Partial tumour regression was the highest in clear cell (26%), less in mixed clear/eosinophilic cell (14%), pure eosinophilic cell (5%) cRCCs and only in 1 of the 31 rhabdoid/sarcomatous tumour showed partial regression. The regressive alterations occurred most frequently in pure clear cell cRCC. In this context, it is important to notice that most clear cell tumours (89%) displayed grade 1, whereas 75% of mixed clear/eosinophilic, 96% of pure eosinophilic tumours displayed nuclear grade 2 or 3 and rhabdoid and sarcomatous cRCCs were grade 3 per definition.

Histology of regressive changes

Most cRCCs showing regressive changes were encapsulated. No complete regression has been found. Regressive areas made up to 30%–90% of tumour mass leaving only a thin rim of tumour cells under the capsule (figure 2A). The regressive areas displayed gradual disappearance of tumour cells and their replacement with tumour-free granulation tissue displaying strong fibrosis in central areas. The histology of regressive areas resembled the inflammatory-proliferative and fibrotic phase of wound healing. Whereby the acute inflammatory stage was not as prominent as in wound healing and overlapped with the proliferative stage. These areas are characterised by inflammatory granulation tissue containing proliferating capillaries, myo-endothelial cells, lymphocytes and macrophages (figure 2B). Towards the central area of regressive alterations, the granulation tissue is gradually remodelled, displayed strong proliferation of naïve activated myofibroblasts (NAF) embedded in light eosinophilic ECM (figure 2C). In the centre of regressive areas, NAF formed a scared tissue that was nearly cell-free and exhibited an increase of collagen fibres (figure 2D).

Immunohistochemistry of regressive tumours

Approximately 30%–50% of cRCC cells under the tumour capsule showed nuclear and membranous expression of TGFB1 in four of the twelve slides analysed, whereas eight cRCC remained negative. Endothels of proliferating small vessels, and macrophages in inflammatory-granulation tissue displayed a strong immunostaining with the TGFB1 antibody (figure 3A). In the fibrotic area, only some NAF and occasionally single macrophages were TGFB1 positive. IL1B was expressed in cells of each of the 12 tumours selected for this study. In the ECM (extracellular matrix) of inflammatory-granulation tissues, dispersed small circular IL1B proteins were seen.

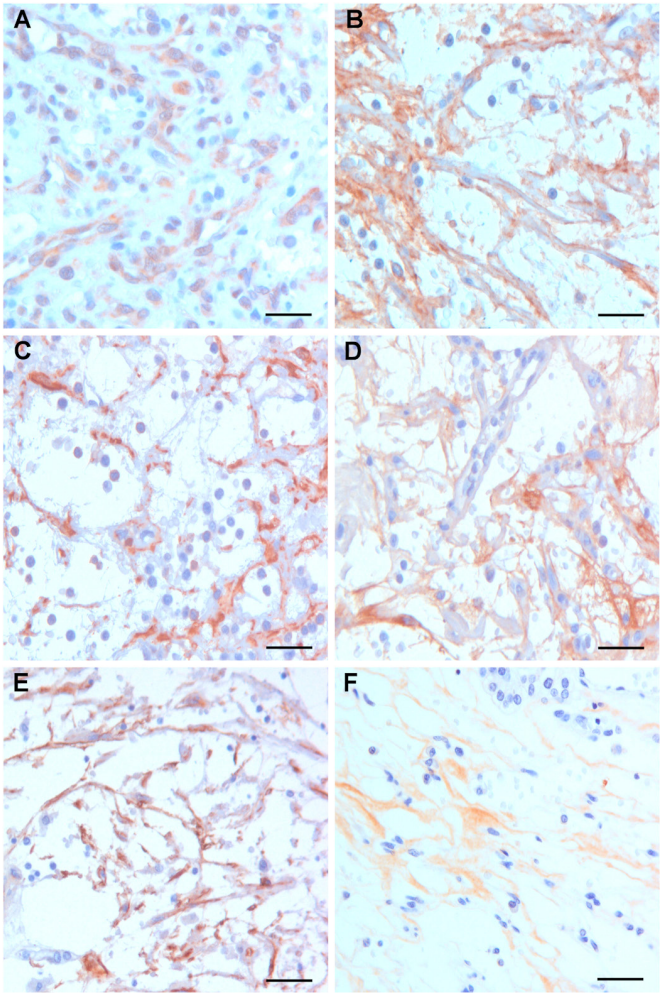

Figure 3 Immunohistochemistry of regressive alterations. (A) Expression of TGFB1 in endothelial cells, endomyo-fibroblasts and macrophages in the inflammation-granulation tissue. (B) Expression of IL6 in myofibroblast and ECM. (C) Expression of myofibroblast marker αSMA in activated fibroblasts. (D) A diffuse FN1 network in the granulation tissue. (E) Parallel running myofibroblasts showing FN1 positive immunoreaction. (F) COL3A1 positive thick collagen fibres in central areas of regressive tissues. Scale bars=40 µm. αSMA, alpha smooth muscle antigen; IL, interleukin, FN1, fibronectin 1, TGFB1, tumour growth factor-beta 1, ECM, extracellular matrix, COL3A1, collagen type I alpha 1, COL3A1, collagen type III alpha 1.

In subcapsular rests of cRCC, IL6 was expressed in stromal fibroblasts and tumour cells remained negative. In the granulation tissue, a diffuse and strong IL6 immunoreaction was seen in NAFs and also in ECM (figure 3B). None of the lymphocytes and macrophages displayed IL6 positive staining. MMP9 (Matrix metallopeptidase 9) was expressed in stromal cells of the cRCC. Moreover, strong MMP9 expression was seen in fibroblasts of cRCC stroma and tumour capsule, and in the transitional area of tumour cells towards granulation tissue. The NAFs in the granulation tissue displayed strong MMP9 expression whereas in central fibrotic areas, no MMP9 expression was seen.

Analyses of the cRCC tissues and regressive areas with αSMA and FAPα, markers of myofibroblasts and inflammatory-fibroblasts, respectively, showed that only αSMA was expressed in the specimens analysed. The thick fibrous tumour capsule showed patchy αSMA expression and vascular-fibroblastic meshwork of cRCC under the capsule were positive for αSMA. In inflammatory-proliferation zone, αSMA positive NAFs were arrayed, akin to tumour area, but instead of tumour cells high number of lymphocytes and macrophages were seen (figure 3C). In the fibrotic areas, most of the αSMA positive NAF were aligned in parallel running order. In the central fibrotic zone, only scattered αSMA positive NAFs were seen. No fibroblast in tumour tissues or regressive areas displayed FAPα positive immunoreaction.

FN1 showed positive staining in the capsule and fibroblastic stroma of cRCCs. In proliferative zone, strong FN1 expression was seen in NAFs, and ECM (figure 3D). In more central areas, FN1 positive NAFs were aligned parallel like αSMA positive NAFs (figure 3E). In the central nearly cell-free fibrotic areas, FN1 displayed a strong fibrillar-homogenous staining. Both COL1A1 and COL3A1 antibodies showed positive immunostaining in highly fibrotic central regressive areas and prevailed parallel organised collagen fibres (figure 3F). COL1A1 and COL3A1 antibodies showed positive immunoreaction in the fibrotic tumour capsule as well.

DISCUSSION

Grading and coagulation necrosis are widely used histological markers to predict the postoperative outcome of cRCC.[13 14] This study showed that partial tumour regression can be added to the prognostic markers. Histological and immunohistochemical data strongly suggest the role of 'wound healing programme' in partial regression of cRCC.[15] Like wound healing, regressive areas of cRCC display inflammatory granulation tissue with neo-angiogenesis, endothelial–mesenchymal cell differentiation, proliferation of NAFs, increased ECM synthesis and alignment of fibrinogen and collagen to provide strength to the central scarring tissue.

An increased expression of TGFB1, IL1B and IL6, which are key cytokines triggering inflammation, was detected in the inflammatory-granulation stage of tumour regression. TGFB1 is critically involved in myofibroblast transdifferentiation and mediates endothelial–mesenchymal transformation leading to accumulation of endo-myofibroblasts.[15–17] In inflammatory-granulation stage, NAF secreted IL6, which recruits TGFB1 secreting macrophages and monocytes. The inflammation potentiates activation of fibroblasts transdifferentiation into myofibroblasts thus promoting tissue desmoplasia in central areas of regression as it was shown in breast and pancreatic cancer.[18 19] In inflammatory-granulation phase of regression, NAFs communicate with ECM via inflammatory cytokines like cancer-associated fibroblasts (CAFs) with cancer cells.[20 21]

Resident fibroblasts in normal kidney keep the balance in tissue homeostasis by permitting only temporary cell proliferation during regeneration. It is possible that in regressive areas of cRCC, the activated fibroblasts restore the homeostatic tissue balance. Under this microenvironmental constraints, reorganisation of ECM to anti-tumorigenic by positive balance of NAF versus CAF in cRCC tissue may restrain tumour growth and results in partial tumour regression.[22] The role of CAF in cancer is quite paradoxical that they can not only support but also restrain tumour development and progression.[23]

Recent investigations indicate that functions attributed to CAF might be executed by different subtypes such as myo-CAF with contractile phenotype and inflammatory CAF regulating tumour-associated inflammation.[24] αSMA and FAPα) are used to identify the subsets of CAF with different gene signature.[25] Recently, it was shown that FAPα is expressed during development of stromal and vascular network in embryonal kidneys, whereas αSMA expressing fibroblasts were found in normal kidney and cRCC stroma.[6] Fibroblasts expressing αSMA mediate contraction and stiffness crosslinking the ECM collagens as it was seen in central cell-free regressive areas of cRCCs.[26]

In proliferation phase of 'wound healing programme', myofibroblasts begin to synthetise and deposit large quantities of ECM proteins including FN1, collagen type I and III, and hyaluronic acid.[21 27] Fibronectin is essential in the initiation of ECM protein assembly including collagen, fibrillin, fibrinogen, fibulin, integrins and thrombospandin and play a role in remodelling of the ECM.[28] The FN1 matrix provides the ground for deposition of other ECM components such as collagens.[29] Fibronectin is essential in initiating the assembly of ECM proteins and plays therefore a role in remodelling of the ECM in wound healing and as it was shown here in the partial regression of cRCC.[28–30] Heterotypic fibrils of collagen 1 and 3 are main fibrillar collagens that are assemled into large mechanically resilient fibres in centrum of tumour regression. Matrix metalloproteases play a fundamental role in ECM degradation in normal physiological processes during embryonal development, wound healing, cancer spreading and are involved in tissue remodelling.[31]

There are remarkable similarities between connective tissue reaction and involvement of fibroblasts in wound healing, end stage kidney disaese, tumour desmoplasia and tumour microenvironment.[32–34] Activated myo-fibroblasts characterised by expression of smooth muscle antigen, αSMA are the main player in these alterations. Stromal fibroblasts in normal kidney, NAF in wound healing, end stage kidneys and CAF in tumours secret αSMA antigen, although they have distinct biological function.[6] Special composition of ECM in each setting, where these fibroblasts reside, may explain the functional heterogeneity of fibroblasts activated in different microenvironment.[24] The fibroblasts remodell their self-generated ECM under different condition and can sustain or promote tumour supporting programmes.[22 23]

Each cRCC with extensive regressive changes was encapsulated with thick fibrotic tissue containing collagen fibres and occasionally lymphocytes. Under the tumour capsule between the tumour cells and fibrotic capsule, MMP9 expression was seen indicating an active biological process. Expression of MMP9 plays a role in progression of several other types of cancer.[31] The histology of tumour capsule remembered to the central, nearly cell-free areas of regressive alterations with parallel organised collagen fibres. The similarity of encapsulation and regressive tumour alteration suggests that encapsulation may be based on response of wound healing as it has been suggested decades ago.[35]

CONCLUSIONS

Many years ago, cancer was referred as a never healing wound suggesting that molecular processes involved in development and progression of cancer are like those occurring during wound healing.[36] The regressive alteration of cRCC parallels the wound healing paradigm of inflammation, proliferation and remodelling.[32] The complex molecular mechanism of crosstalking between cancer cells, fibroblasts and ECM is not well known. Understanding the molecular mechanisms of partial regression of cRCC would be necessary to achieve clinically relevant interventions to restore the antitumour regulation of tumour microenvironment. Recognising the turning point in complex molecular process of shifting the tumorigenic programme to antitumorigenic ones would help to find a way towards 'healing' the never healing wound.

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Contributors All authors contributed to the study conception and design. Material preparation and analysis were performed by LD and GK. Collection of clinical data was performed by DB. The statistical analysis was performed by MY. The first draft of the manuscript was written by LD and DB, and the final version was reviewed by GK. GK is the guarantor and responsible for the overall content.

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Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by Ethics Committee of the University of Pecs, Hungary (No. 8466.PTE 2020). Participants gave informed consent to participate in the study before taking part.

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