

Active caspase 3 and DNA fragmentation as markers for apoptotic cell death in primary and metastatic liver tumours

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Summary

Aims: The induction of tumour cell death by apoptosis is a major goal of cancer therapy and the *in situ* detection of apoptosis in tumour tissue has become an important diagnostic parameter. Different apoptosis detection methods assess distinct biochemical processes in the dying cell. Thus, their direct comparison is mandatory to evaluate their diagnostic value. The aim of this study was to compare the immunohistochemical detection of active caspase 3 and single-stranded DNA in primary and metastatic liver tumours as markers of apoptotic cell death.

Methods: We studied detection of active caspase 3 and single-stranded DNA in 20 primary hepatocellular carcinomas (HCC) and 20 liver metastases from colorectal carcinomas (CRC) using immunohistochemistry on paraffin sections.

Results: Our results reveal that both methods are suitable and sensitive techniques for the *in situ* detection of apoptosis, however, they also demonstrate that immunohistochemistry for active caspase 3 and single-stranded DNA have differential sensitivities in HCC and CRC.

Conclusion: The sensitivity of apoptosis detection using immunohistochemistry for active caspase 3 and single-stranded DNA may be tumour cell type dependent.

Key words: Apoptosis, caspases, DNA fragmentation, hepatocellular carcinoma, colorectal carcinoma, immunohistochemistry.

INTRODUCTION

Most tumours are characterised by increased proliferation, compared with their untransformed cellular counterparts, yielding in a time dependent increase in tumour mass.^{1,2} However, tumour formation is not only determined by excessive cell division and growth but also by reduced apoptotic cell death.^{2,3} Many tumours show increased expression of various anti-apoptotic molecules, which is often paralleled by a down-regulation or inactivation of pro-apoptotic molecules, such as the tumour suppressor p53. Consequently, many tumour cells show an increased resistance to apoptosis induction by a variety of triggers. The importance of apoptosis resistance versus increased proliferation has been recently demonstrated in an elegant study by Pelengaris *et al.*⁴ Transgenic over-expression of

inducible oncogenic c-Myc in the pancreas induces a transient expansion of β -cells, followed by involution due to massive apoptosis induction. In contrast, co-expression of the anti-apoptotic molecule Bcl-2 prevents c-Myc induced apoptosis and results in the development of insulinomas. These data clearly demonstrate the requirement for apoptosis resistance in tumour formation.

Although many tumours show increased apoptosis resistance, signs of apoptosis are frequently observed in solid tumours. *In vivo* apoptosis induction in tumour cells might have different underlying reasons, including insufficient oxygen supply, anti-tumour immune responses, reactive oxygen species, uncontrolled cell cycle, induction of DNA damage, chemotherapy, and many more.² Tumour cell apoptosis stands in contrast to the frequently observed necrotic lesions in the centre of larger solid tumours, most likely caused by insufficient blood supply.

Apoptosis detection and scoring in different tumour samples has become an important parameter in tumour pathology, not only to assess the efficacy of anti-tumour treatment. Typically, apoptotic cell death has been scored histologically, based on the detection of condensed and fragmented nuclei and cells.^{5,6} However, due to the often rapid clearance of apoptotic cells by phagocytic cells,⁷ the rate of tumour cell apoptosis *in situ* might often be underestimated. Thus, more specific methods detecting early events during apoptotic cell death are required for the quantitative scoring of apoptosis.

The detection of DNA fragmentation, characteristic to apoptosis, by TUNEL (terminal deoxynucleotide transferase dUTP nick end labelling) assay has been used to enumerate apoptotic cell death in various studies.^{8,9} However, the TUNEL assay and also other methods detecting DNA fragments have their limitations due to over-fixation artefacts and non-apoptosis-related DNA strand breaks.^{10,11} The biochemical characterisation of the central apoptosis pathways has thus led to the development of novel tools and assays for the detection of apoptotic cell death in solid tissue. Among the most promising are antibodies that recognise apoptosis associated neo-epitopes. Caspases (cystein-aspartate proteases) are central proteases in the apoptosis execution pathway, and are activated by a variety of apoptosis inducing triggers, including chemotherapeutic agents and irradiation.^{12,13} Most apoptosis associated neo-epitopes are the result of caspase activation and their detection is thus

adequately reflecting the induction of apoptotic cell death.¹⁴

In this study, we compared apoptosis detection in 20 primary hepatocellular carcinomas (HCC) and 20 liver metastases from colorectal carcinomas (CRC) by immunohistochemistry using antibodies against (cleaved) active caspase 3 and single-stranded DNA (ssDNA), as a reference and typical marker of apoptotic cell death.¹¹ Both the detection of active caspase 3 and ssDNA were found to be useful methods for the scoring of apoptosis in liver tumours. However, our results also demonstrate that the two methods have differential sensitivity in HCC and CRC.

MATERIAL AND METHODS

Cell lines and media

The human colon carcinoma cell line CaCo2 and the human hepatocellular carcinoma cell line HepG2 were obtained from ATCC and were grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin.

Antibody and reagents

Rabbit monoclonal anti-active caspase 3 antibody was obtained from Cell Signaling Technology (Bioconcept, Switzerland). Anti-ssDNA antibody staining kit was from Alexis (Switzerland). Secondary goat anti-rabbit horse radish peroxidase and goat anti-mouse IgM horse radish peroxidase were from DakoCytomation (Switzerland), goat anti-rabbit FITC and goat-anti-mouse PE were from BD Bioscience (Belgium).

Tumour samples

Paraffin blocks from 20 primary HCC and 20 liver metastases from CRC were used in this study. The surgical specimens employed were obtained from 20 patients with primary HCC who had undergone potentially curative resection, and from 20 patients with primary CRC who had undergone partial hepatectomy due to liver metastases. None of the patients had any previous tumour therapy. Four µm sections were made and the initial diagnosis (HCC or CRC metastases) was confirmed by an experienced pathologist. The clinical information of the different tumour samples is summarised in Table 1. All experiments using human specimens were carried out according to the ethical guidelines of Institute of Pathology, University of Bern, and were reviewed by the institutional review board.

Detection of active caspase 3 by flow cytometry

Active caspase 3 was detected by flow cytometry following the manufacturer's suggested protocol (Cell Signaling Technology, USA). Briefly, CaCo2 and HepG2 cells were seeded in 6-well tissue culture plates and then irradiated with 400 000 and 200 000 µJ UV, respectively, in a Stratalink (Stratagene, USA). At indicated time points, cells were harvested and fixed in 2% paraformaldehyde in PBS for 10 min at 37°C. Then cells were chilled on ice and permeabilised with ice-cold methanol (90% final concentration) for 30 min at 4°C. Unspecific binding was blocked by incubating the cells with PBS containing 5 mg/mL bovine serum albumin for 10 min at room temperature (RT), prior to staining the cells for 30 min at RT with rabbit anti-active caspase 3 (1:100) in blocking buffer. Cells were then washed and

stained with secondary goat anti-rabbit fluorescein isothiocyanate (FITC; 1:200) for 30 min at RT. Finally, cells were washed, fixed in 1% paraformaldehyde in PBS and analysed by flow cytometry on a FACScan using Cell Quest software (BD Biosciences, USA).

Immunohistochemistry for active caspase 3

Four µm sections were cut from HCC and CRC blocks, deparaffinised and stained as previously described.¹⁵ Briefly, sections were pretreated by boiling sections for 10 min in 10 mM citrate pH 6.0 in a pressure cooker to retrieve antigen, and unspecific binding was blocked with 5% goat serum, 1% casein, 0.1% sodium azide in TBS. Liver sections were then stained with a rabbit anti-active caspase 3 antibody (1:100, Cell Signaling Technology) for 1 h at RT. After washing in Tris-buffered saline (TBS), a biotinylated goat-anti-rabbit Ig antiserum (1:500; DakoCytomation) was applied for 1 h at RT. Thereafter, sections were incubated with a streptavidin-biotin-complex/horse radish peroxidase (1:200 in TBS; DakoCytomation) for 45 min. Finally, sections were washed and developed with freshly prepared diaminobenzidine (DAB) solution (Sigma, USA) for 8 min, counterstained with haematoxylin, and mounted. As positive control, sections from nasopharyngeal lymphatic tissue were used; negative controls were performed by omitting the primary antibody.

Detection of ssDNA by flow cytometry

ssDNA was stained as suggested by the manufacturer's protocol (Alexis, Switzerland). Briefly, HepG2 and CaCo2 cells were treated as described above. At indicated time points, cells were harvested and fixed in ice-cold methanol (86% final concentration) overnight. Then cell pellets were resuspended in 50% formamide and incubated for 5 min at RT, followed by 10 min at 75°C. Unspecific binding was blocked by incubation with 3% non-fat dry milk in PBS for 15 min at RT. Cells were then stained with anti-ssDNA antibody (10 µg/ml) for 15 min, washed and counterstained with goat anti-mouse IgM PE (1:200) for 15 min. Finally, cells were analysed by flow cytometry.

Immunohistochemistry for ssDNA

Four µm sections were deparaffinised in xylene, and sequentially rehydrated in 100, 95 and 70% ethanol and PBS. Sections were then incubated in 0.2 mg/mL saponin in PBS for 20 min at RT, washed in PBS and then treated with 20 µg/mL proteinase K in PBS at RT for 20 min. After washing in H₂O, sections were further incubated in 50% formamide at 56°C for 20 min. Sections were then immediately chilled in ice-cold PBS for 5 min, prior to blocking endogenous peroxidase with 3% hydrogen peroxide for 5 min at RT. Sections were rinsed in PBS, blocked for 20 min with 3% non-fat dry milk in PBS, rinsed again with PBS, and incubated with the primary anti-ssDNA antibody (clone F7-26, 10 µg/ml, Apostain, Alexis) at RT for 30 min. After washing, sections were incubated with peroxidase conjugated goat anti-mouse IgM for 30 min at RT. Finally, sections were washed and developed with freshly prepared DAB solution (Sigma) for 8 min, counterstained with haematoxylin, and mounted.

Statistical analysis

The differences in the percentage of positive cells obtained with the two apoptosis detection methods were analysed by Student's *t* test. The correlation coefficient was also determined for tumour size and active caspase 3, tumour size and ssDNA, and active caspase 3 and ssDNA, for each tumour group.

TABLE 1 Clinical data of tumour patients

	Female/Male	Age (years)	Diameter (cm)	Grading
HCC	8/12	62.5 (47–81)	9.18 (1.9–26)	(G2–G3)
CRC	11/9	61.9 (44–73)	5.62 (2–16)	(G1–G3)

Sex, age, size and grading mean values and (range) are shown. HCC, hepatocellular carcinomas; CRC, colorectal carcinomas.

RESULTS

Detection of ssDNA and active caspase 3 in HCC and CRC tumour cells in vitro

In order to validate the two immunohistochemical methods for the detection of apoptosis in HCC and CRC, we initially tested their sensitivity on apoptosis detection in HCC and CRC cell lines *in vitro*. The HCC cell line

HepG2 and the CRC cell line CaCo2 were irradiated with UV light in order to induce apoptosis and cells were harvested at different time points to assess the activation of caspase 3 and the induction of DNA fragmentation. For the better quantification of the signals, stainings were analysed by flow cytometry. Figure 1A shows a typically observed staining for both apoptosis markers. While no increased staining over isotype control was observed in untreated viable cells, a significant shift in fluorescence, identifying specific staining, was observed in UV irradiated cells. The analysis of the kinetic of active caspase 3 and ssDNA staining revealed that both markers increased in both cell lines in a time dependent manner (Fig. 1B) and correlated with the morphological appearance of apoptotic cells (data not shown). Thus, anti-active caspase 3 and anti-ssDNA immunostaining are suitable methods for apoptosis detection in HCC and CRC, and were subsequently used to detect apoptosis in primary and metastatic tumour samples.

Clinical data of tumour patients

The clinical data of the patients and the pathological findings of the 20 HCC and 20 liver metastases of CRC used in the present study are summarised in Table 1. Both

HCC and CRC were re-evaluated with regard to tumour typing, and HCC to Edmondson–Steiner grading. Representative tumour samples were chosen for further analysis of apoptosis by immunohistochemistry.

Detection of active caspase 3 and ssDNA by immunohistochemistry

For the immunohistochemical detection of ssDNA established standard protocols, as provided by the manufacturer, were used. For active caspase 3, different pre-treatment protocols were tested, including proteases, citrate, EDTA and urea treatment. While most pre-treatment resulted in positive staining, antigen retrieval using 10 min pressure cooker in 10 mM citrate pH 6.0 was chosen for subsequent staining based on the best signal-to-background ratio and maintenance of tissue morphology (data not shown). Figure 2 shows representative examples of ssDNA and active caspase 3 immunohistochemistry in HCC and CRC tumour samples. Interestingly, active caspase 3 and ssDNA were detected not only in cells with typical apoptotic morphology (pyknotic or fragmented nucleus, condensed cytoplasm, apoptotic bodies) but also in cells with normal morphology. This staining appears to be specific as control

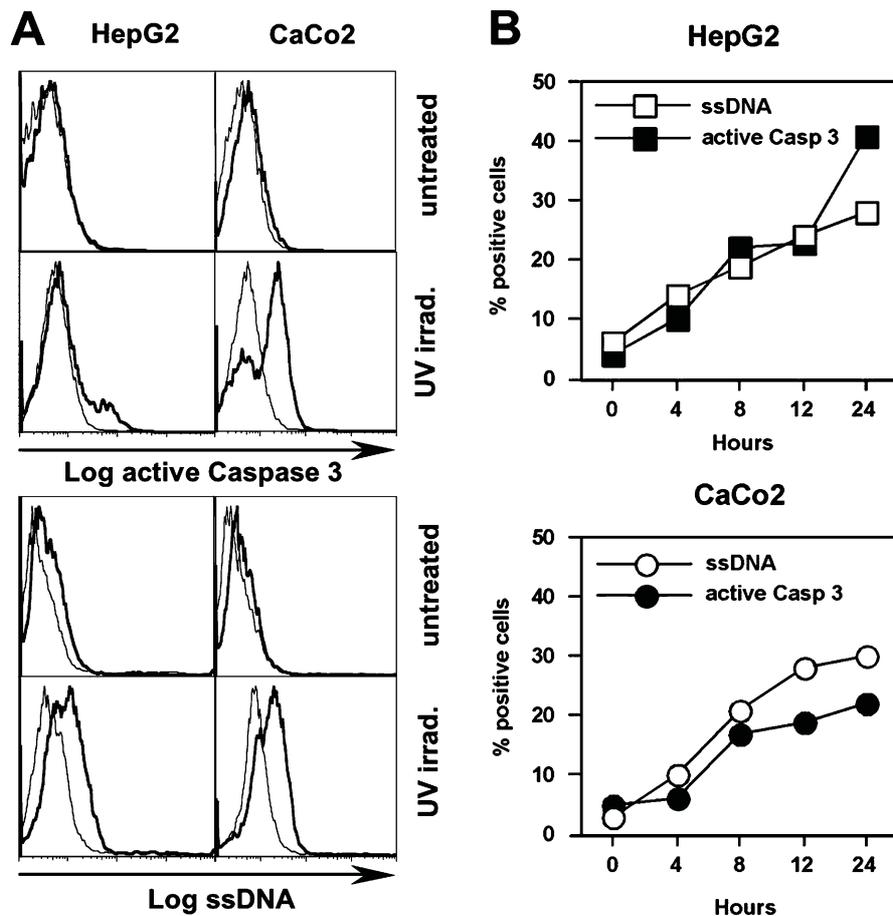


Fig. 1 Detection of active caspase 3 and ssDNA in HepG2 and CaCo2 cells. HepG2 and CaCo2 cells were left untreated or irradiated with UV light to induce apoptosis. (A) Typical staining for active caspase 3 and ssDNA in untreated or apoptotic (8 h post-apoptosis induction) HepG2 and CaCo2 cells. Thick line, specific staining; thin line, control staining. (B) Kinetics of UV induced caspase 3 activation (active caspase 3) and DNA fragmentation (ssDNA) in HepG2 and CaCo2 cells. A typical experiment out of two is shown.

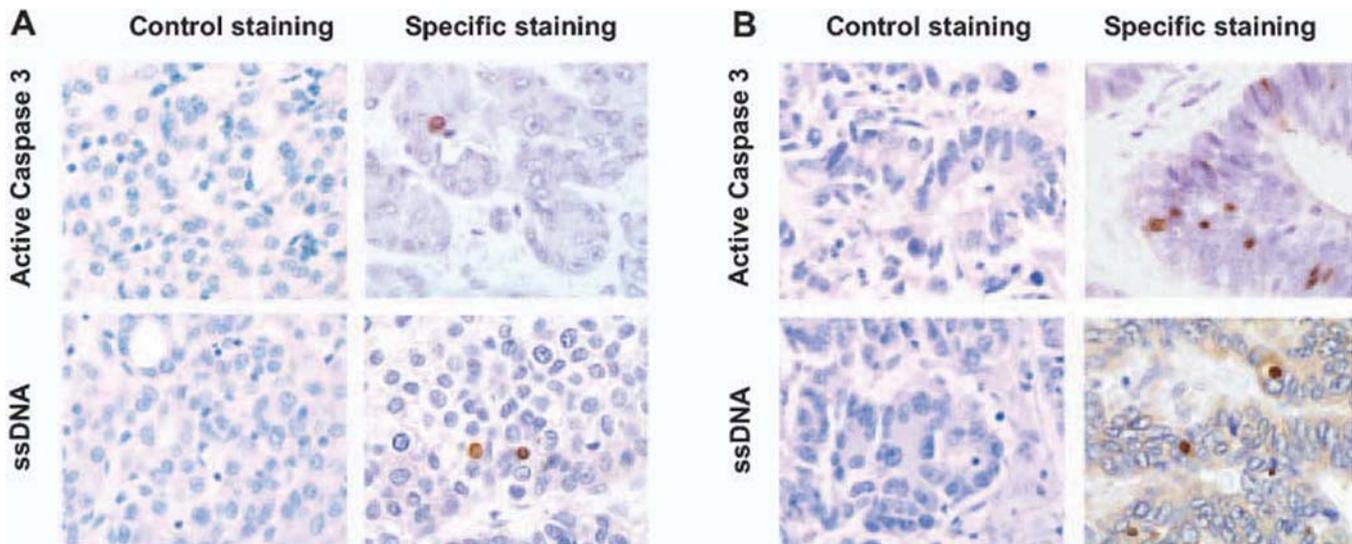


Fig. 2 Immunohistochemical detection of active caspase 3 and ssDNA in HCC and CRC. (A) HCC and (B) CRC were stained with anti-active caspase 3 or anti-ssDNA and corresponding antibody controls. Typical stainings from representative tumour samples are shown ($\times 400$).

staining did not reveal any false positive cells. This indicates that caspase activation and DNA fragmentation may be detectable already at early steps of apoptosis induction without recognisable morphological changes, making them suitable early markers for apoptosis detection in tumour tissues. Importantly, both antibodies did not recognise necrotic cells, abundantly present in both tumour types, demonstrating their specificity for the detection of apoptotic cell death.

Distribution of active caspase 3 and ssDNA positive tumour cells

Positive tumour cells were identified independently of the staining intensity, and the average number of positive tumour cells was determined in at least four randomly selected high power fields ($\times 400$). The number was normalised to total cell number in these areas based on counting individual nuclei. To assess the distribution of apoptotic cells within the tumour tissue, we separately analysed the central area (centre) of the tumour and its invading front (edge) towards the untransformed liver tissue.

Figure 3 shows the mean values of positive cells found in the tumour centre and edge as well as the overall percentage of positive cells, for both HCC and CRC. The data demonstrate a notable increased detection of ssDNA positive cells in the centre of HCC, compared with the tumour edge ($p=0.0024$). While this difference in the distribution of positive cells could also be appreciated using immunohistochemistry for active caspase 3, the accumulation of positive cells in the tumour centre was less pronounced, but significant ($p=0.0006$). These data reflect the idea that HCC tumour cells are exposed to increased cellular stress in the centre of the tumour, resulting in increased apoptotic cell death. Interestingly, no such differential distribution of apoptotic cells within different parts of the tumour was observed in CRC metastases. As shown in Fig. 3, approximately equal numbers of apoptotic cells were found at the tumour edge and in the centre, using both detection methods (active caspase 3 and ssDNA). This suggests that no such increased

stress related apoptosis may be present in the centre of CRC metastases.

Unequal distribution of caspase 3 and ssDNA positive cells in HCC versus CRC

We next aimed to assess the relative sensitivity of apoptosis detection by immunohistochemistry for active caspase 3 versus ssDNA in HCC and CRC. Figure 4 illustrates that only few active caspase 3 positive cells were detected in HCC, regardless of the location within the tumour (edge versus centre). In marked contrast, a significantly higher percentage of positive cells was detected in HCC when employing ssDNA immunohistochemistry ($1.5\% \pm 2.2$ active caspase 3 positive versus $3.9\% \pm 4.2$ ssDNA positive cells, $p=0.029$). As described above, the percentage of positive cells was higher in the centre of HCC than at the edge of the tumours. Thus, ssDNA appears to be the more sensitive marker for apoptosis detection in HCC.

While ssDNA positive cells were also detected in the majority of CRC, immunohistochemistry for active caspase 3 appeared to be considerably more sensitive than that for ssDNA, and about two-fold more positive cells were detected ($9.9\% \pm 4.5$ active caspase 3 positive versus $4.4\% \pm 3.6$ ssDNA positive cells, $p=0.001$). Thus, while ssDNA was found to be the more sensitive marker for apoptosis detection in HCC, with a clear increase of positive cells in the tumour centre, active caspase 3 proved to be the more sensitive marker for cell death detection in CRC.

This trend was also confirmed when HCC and CRC tumours were grouped based on the distribution of active caspase 3 or ssDNA positive cells. Figure 5 shows that while the majority of HCC tumours had only 0–5% active caspase 3 positive cells, most of the CRC tumours had 5–10 or even more than 10% positive cells. In contrast, the distribution of ssDNA positive cells was comparable between HCC and CRC, with the majority of tumours in the group of 0–5% positive cells, and decreasing numbers in the groups of 5–10% or more than 10% positive cells.

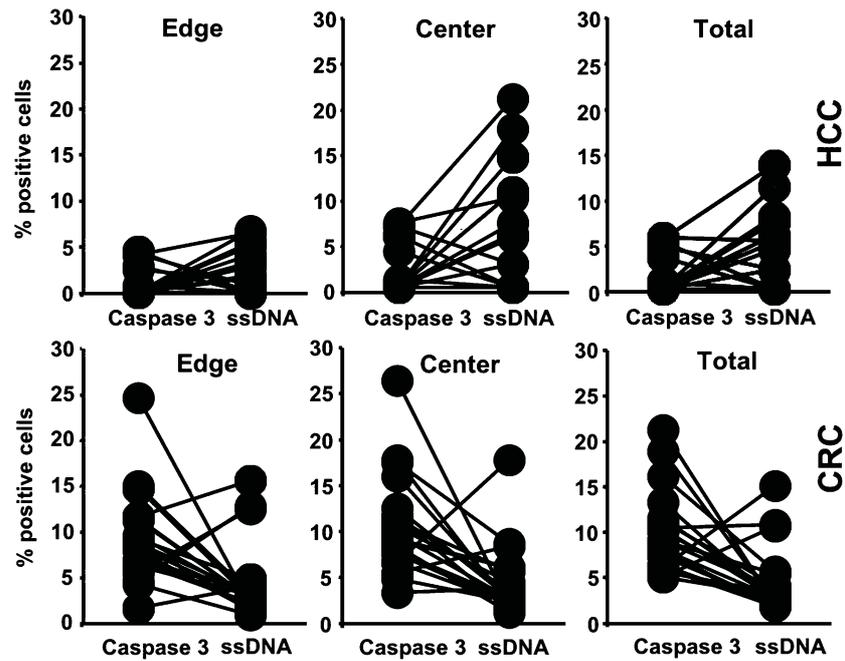


Fig. 3 Distribution of apoptotic cells in HCC and CRC. Twenty HCC and 20 CRC were stained with anti-active caspase 3 or anti-ssDNA antibodies. The percentage of positive cells at the invading front of the tumour (edge), in the central area of the tumour (centre) or in total was calculated. Mean values of each tumour sample are represented by a black bullet, values for active caspase 3 and ssDNA are connected with a line.

Lack of correlation between tumour size and number of apoptotic cells

The data presented above clearly demonstrate that both ssDNA and active caspase 3 are suitable markers for apoptosis detection in tumours, i.e., primary HCC and metastatic CRC in the liver. As the individual tumours in the two different tumour groups varied quite substantially

regarding their size (Table 1; HCC 1.9–26 cm; CRC 2–16 cm) we further aimed to investigate whether tumour size correlates with the number of apoptotic cells. This is of particular interest for two reasons. On one hand it has been suggested that the vascularisation and the nutrient supply of bigger tumours may be limited, leading to increased apoptosis,¹⁶ and on the other hand it may be speculated

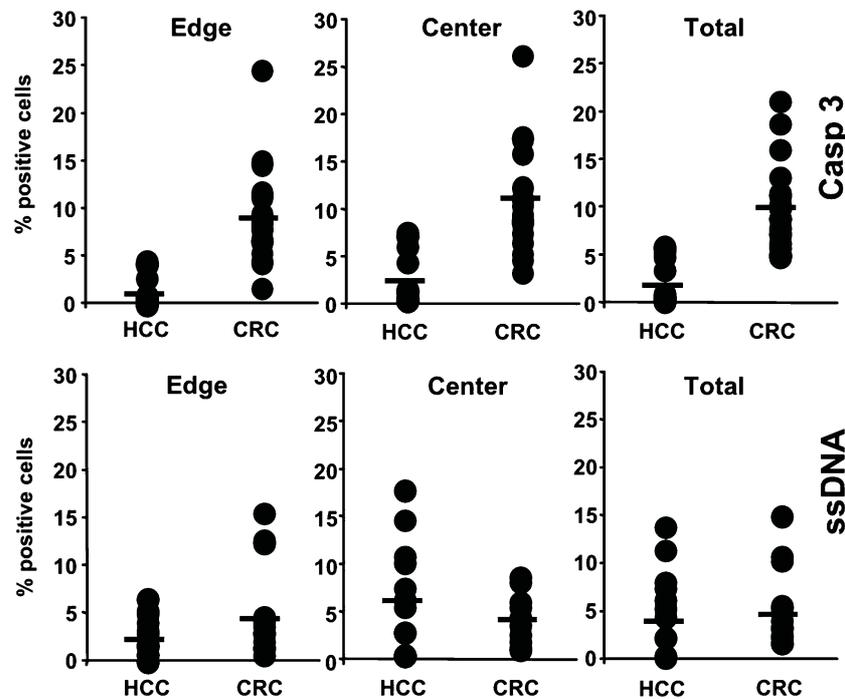


Fig. 4 Comparison between active caspase 3 and ssDNA in HCC versus CRC. The percentage of positive cells for active caspase 3 (Casp 3) or ssDNA of 20 HCC and 20 CRC is shown. Each bullet represents one tumour sample. Bars indicate mean values of all 20 tumour samples. Values at the invading front of the tumour (edge), the central area (centre) or in total are shown.

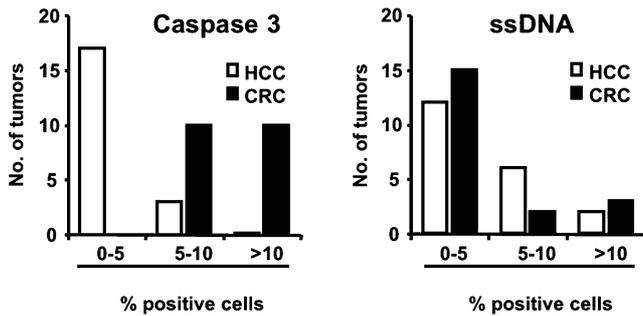


Fig. 5 Unequal distribution of active caspase 3 and ssDNA in HCC and CRC. HCC and CRC tumours were grouped in samples with low (0–5%), intermediate (5–10%) and high (>10%) apoptosis rates. Numbers of tumours per group are shown.

that tumours with increased size may have accumulated tumour cells with additional genetic alterations and increased apoptosis resistance. Surprisingly, we failed to observe any correlation between tumour size of HCC and CRC and the percentage of apoptotic cells (Fig. 6). The lack of correlation was evident using both detection methods, i.e., active caspase 3 and ssDNA (HCC, tumour size versus active caspase 3, $r=0.526$; tumour size versus ssDNA, $r=-0.028$; CRC, tumour size versus active caspase 3, $r=0.108$; tumour size versus ssDNA $r=-0.039$). Besides a certain trend for tumour size versus active caspase 3 in HCC, this analysis suggests that the rate of apoptosis is independent of the tumour size. Although HCC showed a clear increase in the rate of apoptosis in the centre of the tumour versus the edge, also the comparison between tumour size and apoptosis rate in the tumour centre did not result in a significant correlation (data not shown).

We further also analysed whether there was a correlation between the rate of apoptosis detected using the two different immunohistochemical methods. Unexpectedly, we also failed to observe a correlation between the rate of active caspase 3 and ssDNA positive cells in both tumour

groups (HCC, active caspase 3 versus ssDNA, $r=0.120$; CRC, active caspase 3 versus ssDNA, $r=-0.235$).

DISCUSSION

In this study we evaluated, to our knowledge for the first time, the use of anti-active caspase 3 and anti-ssDNA antibodies for the detection of apoptosis in 20 primary and 20 metastatic liver tumours. HCC are among the most frequent tumours and their development is closely related to chronic inflammatory responses in the liver, e.g., hepatitis.¹⁷ Similarly, the liver is the predominant metastatic site for colorectal carcinoma.¹⁸ As apoptosis induction is one of the principle mechanisms of chemotherapy,^{19,20} its detection *in situ* before and after anti-tumour treatment, and its correlation with the expression of pro- and anti-apoptotic molecules, may have an important diagnostic value.

While a broad spectrum of methods is currently available for detection of apoptosis *in vitro*, only a limited number of techniques has been proven suitable for scoring apoptosis *in situ* or, more specifically, in tumour tissue. Even more restricting is the fact that some methods are prone to artefacts (e.g., detection of DNA fragmentation by TUNEL due to non-apoptotic DNA breaks)¹⁰ or are not applicable to certain tissues and tumour types due to the restricted expression of the apoptosis related gene product (e.g., cleavage of the epithelial cell-restricted cytokeratin 18).²¹ In marked contrast, with few exceptions most cell types and tumours express caspase 3, which becomes cleaved and activated upon apoptosis induction. Similarly, DNA fragmentation is observed in (almost) all nucleated cells. Thus, the immunohistochemical detection of active caspase 3 and ssDNA represent two widely applicable methods for the detection of apoptosis in various tissues, including different types of tumours. Our present study has clearly shown that both methods are suitable for detection of apoptosis in primary and metastatic liver tumours.

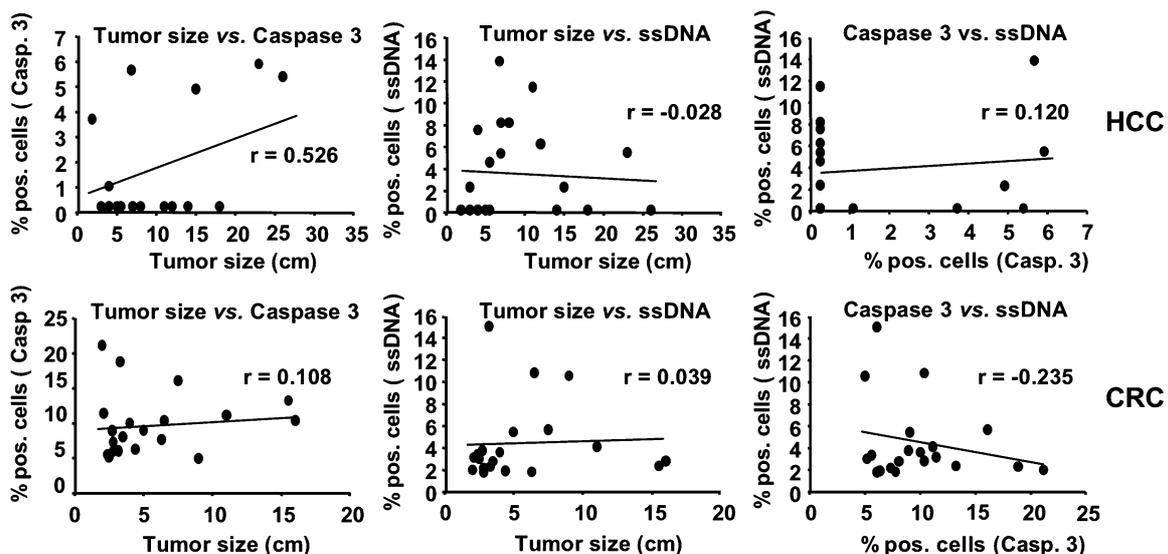


Fig. 6 Correlation between active caspase 3, ssDNA and tumour size. The rate of active caspase 3 or ssDNA positive tumour cells has been calculated for 20 HCC and 20 CRC. Correlation between tumour size and active caspase 3, tumour size and ssDNA, and active caspase 3 and ssDNA has been calculated. r describes the correlation coefficients, lines show the linear best fit curve. Note the lack of correlation between all parameters.

However, our study also revealed that the two different methods are not equally sensitive in the two different tumour groups. Surprisingly, we observed that ssDNA was the more sensitive marker in HCC, while active caspase 3 was more easily detected in CRC. This may have different potential underlying reasons. Caspase 3 activation is thought to represent an early and central step in apoptosis induction by various triggers. In contrast, DNA fragmentation occurs later and is often dependent on caspase 3 activation.^{22,23} Thus, it is likely that in CRC metastases of the liver, active caspase 3 positive cells represent cells at an early step of apoptosis, whereas cells with DNA fragmentation (which is a consequence of caspase activation) may already be expelled from the epithelial layer into the lumen.^{24,25} We frequently observed immunoreactive apoptotic cells or cellular aggregates in the glandular lumina of neoplastic glands of CRC, most likely representing an accumulation of detached late apoptotic cells. In marked contrast to CRC, we found that ssDNA was the more sensitive marker for apoptotic cell death in HCC. As apoptotic cells cannot be expelled from the tumour tissue and phagocytosis of dying cells by neighbouring tumour cells may be limited,²⁶ the elevated number of ssDNA-positive cells in HCC could represent an accumulation of late apoptotic cells. Interestingly, we failed to observe a correlation between tumour size and number of apoptotic cells, using both methods. This is surprising, as it is likely that the nutrient supply and therefore cellular stress is impaired in bigger size tumours than in smaller ones. A possible explanation of our finding, i.e., lack of correlation between tumour size and apoptosis frequency, therefore may be that very small tumours have not been included in our study.

While both methods were found to reliably detect apoptotic cell death in HCC and CRC metastases, the lack of any correlation between the two different methods was surprising. One possible explanation may be that the very low detection of apoptosis in HCC using anti-active caspase 3, and in CRC using anti-ssDNA, respectively, may obscure the relationship with the more abundantly detected apoptosis marker. Importantly, however, our study illustrates that active caspase 3 and ssDNA immunohistochemistry are not equally well suited for apoptosis detection in different types of tumours, and thus are also not directly comparable. These findings may have important implications for future studies on apoptosis detection in tumour tissue from different cellular origin.

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