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Carbonic Anhydrase IX (CAIX) Expressing Hypoxic Micro-environment Hampers CD8+ Immune Cell Infiltrate in Breast Carcinoma

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Background: Hypoxia and necrosis are common features of invasive cancer. The dynamic upregulation of carbonic anhydrase IX (CAIX), triggered by hypoxia-inducible factor 1 (HIF-1) is 1 of the mechanisms supporting cellular adaptation to hypoxia in solid tumors, including breast carcinoma. CAIX activity results in extracellular acidosis and in a profound reorganization of the tumor micro-environment, influencing biological behavior and prognosis. The main focus of our study was to evaluate the mass and distribution of the immune infiltrate, more specifically of CD8+ effector T-cells, in relation with tumoral CAIX expression.

Materials and Methods: Formalin-fixed and paraffin-embedded breast carcinoma sections were analyzed following double immunohistochemical staining for CAIX and CD8. Scanned digital slides were evaluated for both labelings, and CD8-related signal was determined within and outside CAIX-positive tumor areas using the HistoQuant (3DHistech) image analysis software. Statistical analysis was performed using GraphPad Prism software.

Results: Of the 34 breast carcinomas, 18 tested partially positive for CAIX. The remaining 16 cases were used as the CAIX-negative control group. Necrotic foci were generally associated with CAIX overexpression, and tumors exhibiting signs of necrosis had a significantly higher rate of relative CAIX expression compared with samples without necrosis (11.47 ± 5.505 vs. without necrosis 3.765 ± 3.5 P-value $= 0.0216$). On the other hand, no statistically significant difference was found when comparing relative CD8+ lymphocyte counts in cases with necrosis as opposed to those where necrosis was absent (134.7 ± 55.7 vs. 97.70 ± 57.25; P value $= 0.1579$). No difference in gross CD8+ T-lymphocyte infiltrate could be measured between CAIX positive and negative samples (98.48 ± 37.32 vs. 95.99 ± 50 P value $= 0.5928$). However, in CAIX-expressing tumors a statistical correlation between the CD8+ T-lymphocyte infiltrate and the extent of CAIX-positive areas was observed. Within the same tumor, CD8+ T-lymphocyte counts showed a significant difference betweeen CAIX+ and CAIX- areas (13.06 ± 9.4 vs. 135.6 ± 62.2 P value <0.0001).

Conclusion: Our measurements demonstrate for the first time that tumor areas with CAIX expression potentially hamper CD8+ T-lymphocyte infiltration in breast carcinoma. The hypoxia-driven adaptive micro-environment likely interferes with the specific response to biological and immune therapies requiring intact effector T-cell response.

Key Words: CAIX, micro-environment, breast cancer, tumor-infiltrating lymphocytes (TIL)

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Relative hypoperfusion results in hypoxic areas in fast-growing malignant tumors. To survive, cancer cells gain the ability to adapt to hypoxic stress through the reprogramming of their homeostasis and cellular metabolism and their immediate environment, mainly coordinated by the activation of hypoxia-inducible factor HIF-1.[1] On the other hand, necrosis caused by tumor ischemia is a common feature of aggressive cancer, indicating the failure of the adaptive program. Necrotic foci have traditionally been attributed to insufficient vascular supply in rapidly growing cases.[2] Several studies have shown that breast carcinomas showing intratumor necrosis generally have a worse prognosis, are often estrogen and progesterone receptor negative, and are featured by high histologic grade, lymphatic, and distant organ metastasis.[3–5] As such, the occurrence of necrosis as an unfavorable feature should be incorporated into the standardized histopathological report.

In general, tumor hypoxia might be far more extended than it is suggested by the mass of necrosis. Adaptive changes contributing to cancer cell survival can also be demonstrated at the level of histology. Carbonic Anhydrase IX (CAIX) expression, triggered by hypoxia, is an established prognostic marker in solid tumors. Clinical studies have shown that the rate of CAIX expression in

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solid tumors correlates with therapy resistance.[6,7] Carbonic Anhydrases (CA) principally catalyze the reversible transformation of CO_2 that contributes to the neutralization of the elevated intracellular H+ ion concentration caused by hypoxia-induced glycolysis.[8–10] The resulting extracellular acidosis drastically modifies the micro-environment by enhancing the activity of certain proteases, matrix degradation, and angiogenesis. Moreover, acidic pH-related interaction of stromal and immune cells may impair anti-tumor defense in the proximity of the affected malignant cell groups. The access of certain anti-tumor drugs, therapeutic antibodies, and immune cells to their targets may also be blocked this way.[11,12]

The effect of CAIX expression in breast carcinoma was extensively investigated in the past, including several clinical studies along with both in vitro and in vivo observations. Experimental in vitro data show that the proliferation and metastatic ability of the tumor can actively be decreased by selective inhibition of cancer-related CA-s.[13] CAIX activity measured by in vivo imaging methods became a promising variable to be utilized as a biomarker reflecting tumor metabolism.[14] According to clinical studies, the rate of CAIX expression also correlates with the overall size and the invasive nature of the tumor.[12,15,16] However, no detailed studies were made on how CAIX expression affects tumor-stromal and immune interactions in breast carcinoma. Our goal was to find out whether the intratumoral expression of CAIX and its extent affects the distribution and quantity of immune cells. For this purpose, a digital slide-based image analysis approach was elaborated. According to our procedure, the quantity of CD8+ lymphocytes—as a prototype for immune effector cells—could be measured and compared between CAIX expressing and non-CAIX-expressing tumor areas following double immunohistochemistry (IHC). Our results indicate to a significant intratumoral variability in CD8+ cell density in relation to CAIX expression in breast carcinoma.

MATERIALS AND METHODS

A probe set of 34 cases of invasive breast carcinomas were selected from the archives of the Department of Pathology, Debrecen, University Clinical Center and the Department of Pathology, Kenézy Hospital, Debrecen, from the period between 2018 and 2021. The work was approved by TUKEB (Hungarian National Bioethical Research Committee, 60355-2/2016/EKU). Necrosis, as a reliable marker of hypoxia was present in 1 group of the tumor samples; the remaining cases (without necrosis) were used as a control group. Samples from primary invasive breast carcinomas of no special histologic type were included, and neoadjuvant therapy was not applied. Patients were between 35 and 84 years old. All tumors were classified as grade 2 or 3, measuring between 14 and 110 mm-s. Tumor stages with respect to the individual tumor varied from pT1c to pT4b.

Major tissue variables and the occurrence and degree of tumor necrosis were determined using conventional HE-stained sections. Samples were classified as CAIX+ or CAIX− according to the presence of conventional CAIX immunostaining on 4 um thick sections of formalin-fixed, paraffin embedded histologic samples. Further, double CAIX and CD8 immunohistochemical staining were performed. The staining was done after validation of the protocol for a BenchMark automated immunostaining system (Ventana-Roche). To highlight CAIX the Carbonic Anhydrase IX (EP161) rabbit monoclonal antibody clone 379R-16 (Cell Marque Corporation) (1/200 dilution) and the OPTIVIEW DAB detection kit (Ventana-Roche) was used. For CD8 IHC we used the mouse antibody clone C8/144BB (M710301-2, Dako-Agilent Technologies Company) (1/400 dilution) and the Universal Alkaline Phosphatase Red detection kit (Ventana-Roche). The stained slides were digitalized using a Pannoramic Midi (3DHISTECH Kft.) slide scanner (single layer, focus position: 1600, quality: 80, bit depth: 8-bit good image quality, large file size, fine quality settings). After scanning, we used the Pannoramic Viewer software for the selection of CAIX positive and CAIX negative areas as well as for CD8 positive T-lymphocyte counting (HistoQuant Module V36972J). Following the annotation of CAIX-positive and CAIX-negative areas, the size of the areas was calculated by the software in mm^2. To evaluate the cases, we examined occasionally 1-1 standard slides, on which a total of 9 mm^2 was annotated. The software identified CD8+ (red) cells based on color segmentation which was not hindered by CAIX+ (brown background) staining. Manual cell counting was also done as a control for software operation. Cell recognition and counting were done by using Blur (Gauss) noise reduction level 32. Color spectrum calibration required multiple manual sampling, which defined the following spectrum: Hue: 278-330, Sat: 15-65. Also, manual filter settings were used to determine signal size. The software used units to define object size (cells). The smallest object captured was 0,01 units, while the largest object acceptable as a single cell was 646,13 units. Objects below 25 units (staining artefacts, cell debris, etc.) were excluded from the evaluation. The overall cell quantities measured were in good agreement with the cell number counted manually. Absolute CD8+ lymphocyte counts measured were then converted into relative values (per 1 mm^2 tumor area) by dividing with the total area of measurement. Statistical analysis was done with the Prism 5 for Windows software, Version 5.03 (GraphPad Software Inc.).

RESULTS

Altogether 34 female breast carcinoma samples were enrolled in the study. The amount of CAIX expressing tumor mass was extremely variable in the 18 samples defined as positive. As expected, perinecrotic areas identified by morphology were strongly associated with CAIX overexpression. However, CAIX+ areas frequently appeared also in the absence of necrosis, directing to the dynamic nature of hypoxia-driven upregulation. The successfully executed double immunohistochemical staining perfectly demonstrated the relationship between CAIX

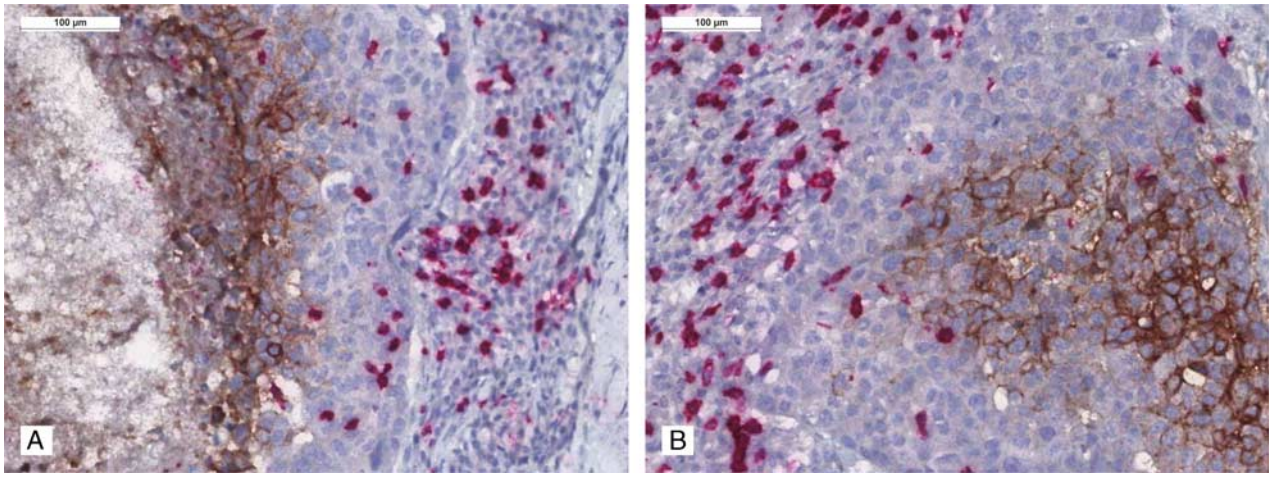

FIGURE 1. A, Tumoral necrosis in invasive breast carcinoma, double-stained for CAIX (DAB/brown) and CD8 (Fast-Red/red). Intratumoral hypoxic compartments are clearly distinguishable from left to right: necrosis (amorphous zone), CAIX positive adaptive zone (brown area, virtually lacking red stained lymphocytes), intact tumor zone (no brown staining, with several tumor-infiltrating CD8+ lymphocytes) and tumor stroma (with masses of peritumoral lymphocytes, including high numbers of CD8+ T-cells)(20× magnification); B, Area of invasive breast carcinoma without necrosis, but CAIX+ central hypoxic zone (brown labeling) surrounded by the intact tumor zone (few CD8+ T-cells) and the lymphocyte-rich tumor stroma (plenty of CD8+ cells, left). The image reflects a typical cellular distribution; the difference in the CD8+ lymphocyte frequency was evident in the majority of the samples (20× magnification). CAIX indicates carbonic anhydrase IX.

and CD8-positive lymphocytes. (Figs. 1A and B) To evaluate the distribution of tumor infiltrating immune cells in the context of CAIX positivity, sections double stained for CAIX and CD8 were analyzed with digital image processing. CD8+ T-cells highlighted in a dark red color (Fast-Red) could be easily identified and digitally captured inside and outside CAIX+ areas (DAB, brown staining) (Figs. 2A and B). Automated segmentation on CD8+ labeled cells also enabled the presentation of all positive events in an image gallery for further verification, allowing the removal of unclear objects from further calculations. This unique approach enabled an objective comparison of CD8-labelled T-cell counts between individual samples and between different regions, defined as CAIX+ and CAIX- areas of the same sample.

The detailed evaluation of the sample cohort identified 18/34 samples (53%) with CAIX-positive areas by IHC. Immunopositivity was always focal, and a strong cell membrane-bound reaction was seen in most of the samples. The remaining cases (16/34, 47%) lacking specific CAIX IHC signal made the negative control group. Necrosis was present in 17/34 cases (50%). We observed CAIX positive status accompanied by necrosis in 12 cases, while CAIX negative status and lack of necrosis were seen

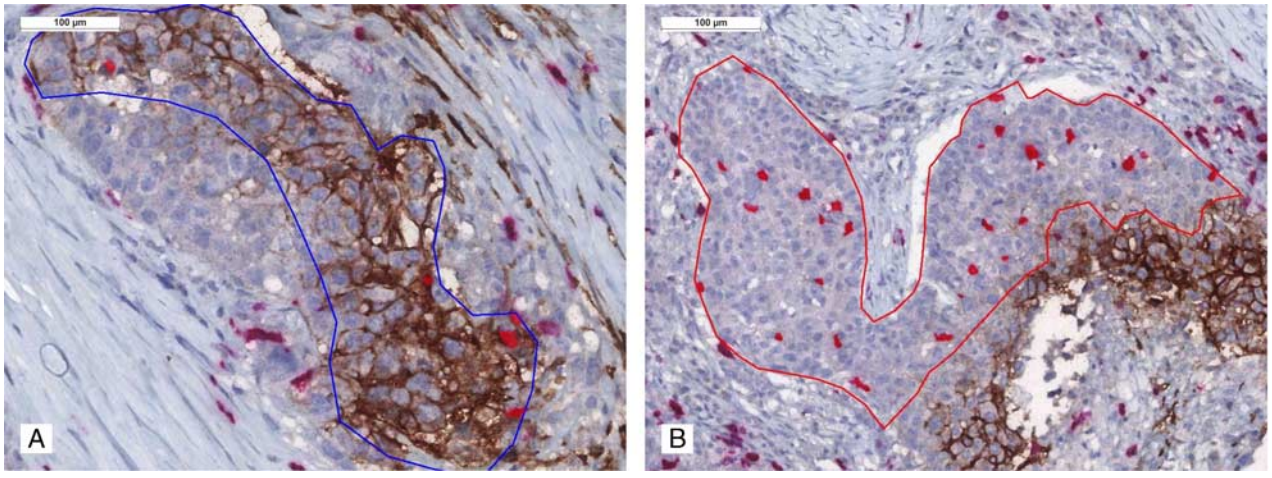

FIGURE 2. Digital image analysis of CAIX and CD8 double-stained IHC sections following the annotation for CAIX positive and negative tumor regions. A, CAIX positive region (blue line) with very modest CD8+ infiltrate, digitally marked as individual red objects (20× magnification); B, CAIX negative tumor region (red line) with uniformly distributed red objects referring to intratumoral CD8+ lymphocytes (20× magnification). CAIX indicates carbonic anhydrase IX.

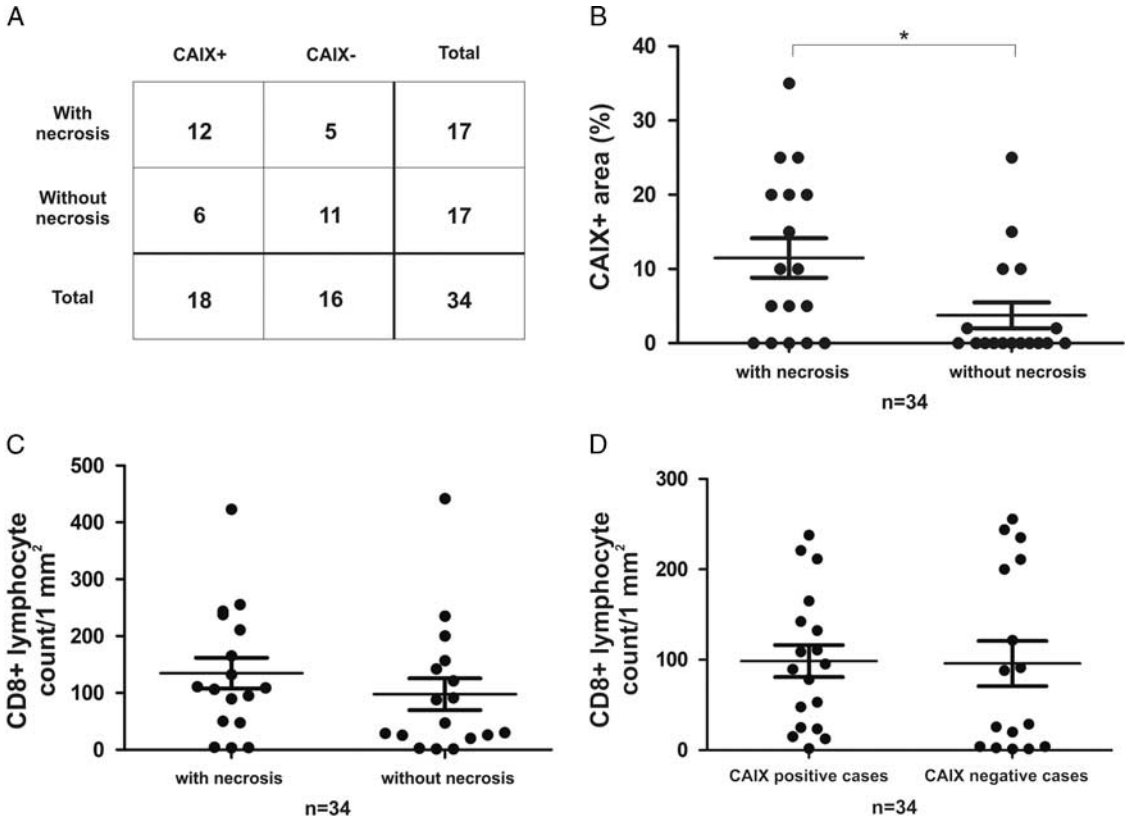

FIGURE 3. Necrosis and intratumoral CD8+ cell counts due to CAIX-positivity in breast carcinoma. A, Summary table of the frequency of included cases (n = 34) concerning necrosis and CAIX expression. B, The extent of CAIX-expression in relation with tumor necrosis observed by histology in invasive breast carcinoma. CAIX-expression between tumor groups with and without necrosis proved to be significantly different (*$P = 0.0216$) C, No difference in relative CD8+ count could be measured between tumors with and without necrosis ($P = 0.1579$) D, No statistical difference in relative CD8+-frequency could be stated between CAIX-positive and CAIX-negative carcinomas ($P = 0.5928$). CAIX indicates carbonic anhydrase IX.

in eleven cases. In 6 cases, we observed CAIX positive status without tumoral necrosis, and in 5 cases, CAIX negative status despite the presence of necrosis (Fig. 3A).

The relative CAIX expression could be determined in digital slides by the HistoQuant image analysis application. The total of CAIX+ areas was selected and individually measured within each slide and its proportion to the total tumor area was calculated in percentage. The extent of CAIX+ area/tumor area within individual sections ranged between 2.0% to 35.0%. First, we looked at the association between relative CAIX expression and tumor necrosis as potentially converging consequences of tumor hypoxia. In those tumors where necrosis was uncovered morphologically, the relative CAIX expression appeared to be significantly more prevalent compared with the nonnecrotic tumors (mean CAIX% with necrosis 11.47 ± 5.505 vs. without necrosis 3.765 ± 3.5 P- value = 0.0216, Mann-Whitney test) (Fig. 3B).

Both necrosis and CAIX-labeling were further evaluated in the context of lymphocytic infiltrate. When comparing CD8+ T-cell counts between tumors with and without necrosis, no significant difference was observed even after removing outliers (with necrosis 134.7 ± 55.7 vs. without necrosis 97.70 ± 57.25 P value = 0.1579; Mann-Whitney test) (Fig. 3C).

The measurement of CD8+ counts in individual samples with or without CAIX expression could be uniformly performed by focusing exclusively on the red IHC signal. The result was displayed as the relative lymphocyte count/mm^2 tumor area. This comparison did not show any gross inter-tumoral difference in the CD8+ frequency (CAIX+ areas 98.48 ± 37.32 vs. CAIX- areas 95.99 ± 50 P value = 0.5928; Mann-Whitney test) (Fig. 3D).

As next, a detailed analysis of the 18 samples with variable amounts of CAIX-positivity followed. We correlated relative CD8+ lymphocyte counts with the total extent of CAIX-positive areas measured in the individual samples. The CD8+ lymphocyte count proved to be highly variable and did not correlate with the area of the CAIX labeling (Spearman correlation R square = 0.05388, no statistically significant difference) (Fig. 4).

Intratumoral CD8+ distribution was defined following the separate measurement of individual CAIX+ and CAIX− tumor areas within the same tumor section. The mean area size was 0.07 ± 0.04 mm^2 (range between 0.001 and 0.76 mm^2, altogether 710 annotated areas were

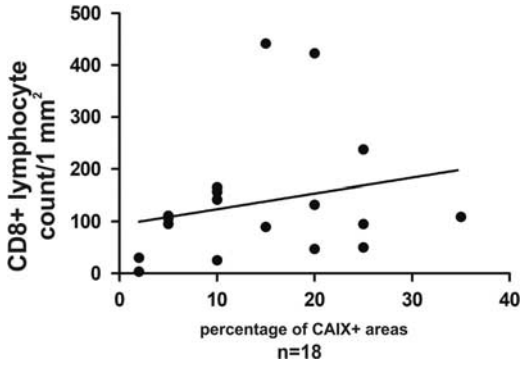

FIGURE 4. Comparison between the extent of CAIX expression and relative CD8+ counts in breast carcinomas with CAIX-expression (n = 18). No correlation was found. (Spearman correlation, R square: 0.05388, after removing outliers). CAIX indicates carbonic anhydrase IX.

measured). In support of our expectations and the morphologic observations, the relative number of CD8+ cells varied in a regular fashion: in the CAIX-negative tumor compartment CD8+ cell counts highly exceeded one of the CAIX-positive areas of the same tumor sample (CAIX- areas 170.8 ± 67.8 vs. CAIX+ areas 13.06 ± 9.4). Using the Mann-Whitney test, we obtained a statistically highly significant difference in the relative CD8+ T-lymphocyte frequency when CAIX-positive and negative tumor areas were compared (P value < 0.0001) (Fig. 5).

DISCUSSION

CAIX activation, induced by HIF-1, is 1 of the prominent adaptive mechanisms to restore intracellular pH-balance evolving in hypoxia. The mass exclusion of protons to the extracellular space assumes a profound biochemical effect on the micro-environment surrounding the hypoxic tumor area. The use of specific antibodies enables the simple detection and quantification of cancer-related CAIX expression in histologic conditions, providing a dynamic biomarker. Because of the differences in tissue perfusion, the heterogenous distribution of

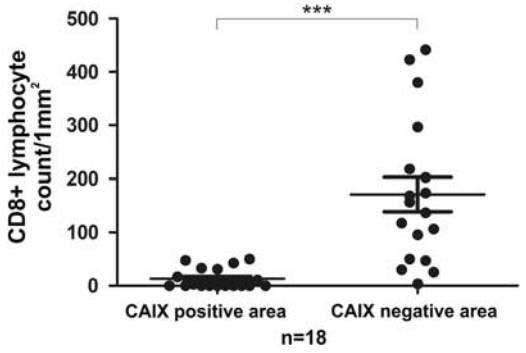

FIGURE 5. Cumulative plot presenting intratumoral CD8+ cell distribution in CAIX positive and negative areas of CAIX-expressing breast carcinomas (n = 18). CAIX expression is associated with the local suppression of immune cell infiltrate compared with CAIX-negative areas n = 18 (***P < 0.0001). CAIX indicates carbonic anhydrase IX.

CAIX in tumor samples was repeatedly reported. During our studies, we could confirm the correlation between necrotic tumor foci and zonal CAIX overexpression using immunohistochemistry. CAIX-positive areas lacking obvious necrosis were consistent with sub-lethal adaptive changes due to hypoxia. CAIX-driven H+-ion excretion and related extracellular acidosis might interfere with key biological mechanisms in a currently underestimated fashion, including the migration and specific anti-tumor action of otherwise intact immune cells.[17] The amount of tumor-infiltrating lymphocytes (TIL) is an important prognostic marker in different cancer types, such as colon, ovarian, lung, and breast carcinoma.[18–21] More specifically, the amount of TIL is associated with both disease prognosis and therapeutic efficacy, as stated for breast carcinoma.[21,22] However, the actual amount and composition of the immune infiltration are highly variable and, thus, difficult to consider. CD4+ T-helper1 (Th1) cells support the process of antigen presentation by cytokine secretion and the activation of antigen-presenting cells, while CD8+ cytotoxic T-cells (CTL) are responsible for eradicating tumor cells.[23] According to some recent findings, higher CD8+ T cell infiltration is generally associated with better prognosis and response to therapy.[24,25] Breast carcinomas occurring at a young age and triple-negative breast cancers showed higher TIL values and high programmed death-ligand 1 (PD-L1) expression,[26] saying that the amount and composition of the immune infiltration is an evolving clinical feature in invasive breast cancer.[27] The main question of our study was how far the variation of hypoxia-related CAIX expression interferes with the amount and distribution of intra-tumoral immune infiltrate and whether and how these proposed differences can be measured. We were specifically focussing on the CD8+ effector T-cell compartment of the immune cells as a population with clear functionality and optimal visibility by immunohistochemistry. To address the question of quantification, we applied double IHC stained scanned histologic slides and a digital image analysis approach. The method relied on the identification of specifically marked CD8+ T-lymphocytes within tumor areas predefined according to their CAIX expression. The HistoQuant module enabled the quantification of the objects in 2 separate color channels, and the number and size of the objects could be obtained. Measurement parameters were provided in a simple numeric format for further analysis and statistical calculations. Thus, digital image analysis of real-life tumor samples gave us a deep insight into the actual distribution of CD8+ cells in the special context of hypoxia-related CAIX-overexpression.

In general, CAIX was associated with the presence of necrotic tumor foci; however, strong staining areas were also seen without visible tissue damage, most probably reflecting sub-lethal adaptive zones of the tumor. Although CAIX expression correlates with the presence of hypoxia and/or tissue necrosis according to the consensus of the literature, the extent of hypoperfusion may vary considerably and does not necessarily affect larger tissue masses. In agreement with this, a broad range of CAIX expression could be measured in this breast carcinoma cohort. CAIX-positive adaptive areas often represent the minority of the tumor without a measurable effect on other associated variables, such as the overall CD8+

frequency. Accordingly, the relative rate of intratumoral CD8 + lymphocytes did not differ in general between breast carcinomas with and without CAIX-expression, which may rely on mass effect besides many other, yet undefined factors.

In contrast, we could demonstrate a significant difference in CD8+ cell distribution within breast carcinoma samples displaying CAIX expression, when CAIX-positive and CAIX-negative areas of the same tumor were measured following digital separation. Even more exciting was that a clear negative correlation could be stated: CAIX-positive areas presented with significantly less immune cells, while CAIX negative areas showed much higher numbers (5 to 10-fold difference) CD8+ tumor-infiltrating lymphocytes. This reverse relation suggests that the hypoxic, CAIX-expressing environment unfavors the migration of effector CD8+ cells, which is otherwise enabled in the absence of CAIX activity within the same sample.

According to the general view, CAIX, together with other factors, is a sensitive adaptive mechanism induced by hypoxia in different kinds of malignancies. CAIX activity contributes to extracellular acidosis and, thus, has the potential to restrict intratumoral immune cell migration. TIL, also covering masses of CD8+ effector T-cells, is a complex cellular compartment,[28,29] which appears to be highly variable in tissue samples, directing to the importance of the local microenvironment. As a major factor, hypoxia induces basic reprogramming with a major effect on the extracellular milieau. The extrusion of CD8+ T-cells in the CAIX+ hypoxic zone, shown herein, is in line with the idea of enhanced immune resistance and survival of tumor cells adapted to acidosis and hypoxia.[30,31] According to the presented data, we presume that the ability of immune cells to invade the tumor is zonally variable, and low accessibility zones typically express CAIX. Further, CAIX-immunostaining, which is simple to perform and measure, may highlight immune-protected areas as a dynamically formed specific feature of the developing tumor.

In this study, we describe a method based on double IHC staining and digital image analysis, keeping in mind that the preliminary results should have to be verified in much larger cohorts, including detailed tumor classification. The presented method appears to be practical enough to be utilized for a broad spectrum of cancers and allows to determine immune infiltrates in a wide variety of functionally/phenotypically different areas of the same sample. Our data clearly show how local changes of the micro-environment affect intratumoral immune cell distribution, thus influencing the natural progression and, most probably, the therapeutic response of the malignant disease.

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