

Predictive Biomarkers and Personalized Medicine

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A Comprehensive Analysis of Human Gene Expression Profiles Identifies Stromal Immunoglobulin κ C as a Compatible Prognostic Marker in Human Solid Tumors

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Abstract

Purpose: Although the central role of the immune system for tumor prognosis is generally accepted, a single robust marker is not yet available.

Experimental Design: On the basis of receiver operating characteristic analyses, robust markers were identified from a 60-gene B cell-derived metagene and analyzed in gene expression profiles of 1,810 breast cancer; 1,056 non-small cell lung carcinoma (NSCLC); 513 colorectal; and 426 ovarian cancer patients. Protein and RNA levels were examined in paraffin-embedded tissue of 330 breast cancer patients. The cell types were identified with immunohistochemical costaining and confocal fluorescence microscopy.

Results: We identified immunoglobulin κ C (IGKC) which as a single marker is similarly predictive and prognostic as the entire B-cell metagene. IGKC was consistently associated with metastasis-free survival across different molecular subtypes in node-negative breast cancer ($n = 965$) and predicted response to anthracycline-based neoadjuvant chemotherapy ($n = 845$; $P < 0.001$). In addition, IGKC gene expression was prognostic in NSCLC and colorectal cancer. No association was observed in ovarian cancer. IGKC protein expression was significantly associated with survival in paraffin-embedded tissues of 330 breast cancer patients. Tumor-infiltrating plasma cells were identified as the source of IGKC expression.

Conclusion: Our findings provide IGKC as a novel diagnostic marker for risk stratification in human cancer and support concepts to exploit the humoral immune response for anticancer therapy. It could be validated in several independent cohorts and carried out similarly well in RNA from fresh frozen as well as from paraffin tissue and on protein level by immunostaining. *Clin Cancer Res*; 18(9); 1–9. ©2012 AACR.

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Translational Relevance

This study reports that immunoglobulin κ C (IGKC) RNA levels robustly define prognosis in a comprehensive analysis of available breast cancer data sets and predict response to neoadjuvant anthracycline-based therapy. In addition, IGKC maintains its prognostic relevance also in non-small cell lung and colorectal cancer, suggesting a global mechanism in the biology of adenocarcinomas. Using real-time PCR and immunohistochemistry in formalin-fixed, paraffin-embedded (FFPE) tissue, we could validate the prognostic impact of IGKC. Furthermore, using confocal microscopy, we identified tumor-infiltrating plasmablasts and plasma cells as the source of IGKC expression. This study has major clinical implications: (i) IGKC as a prognostic and predictive marker that lends itself to systematic testing in FFPE tissue samples allows an improved prediction of prognosis and response to chemotherapy, and (ii) the protective effects of this naturally occurring humoral immune response support the concept of immunotherapy.

Introduction

It has become evident that the immune response in the tumor environment plays a pivotal role in all stages of carcinogenesis and in different context may promote or inhibit tumor progression (1–3). In human cancer, this concept is supported by the observation that the presence of specific immune cells can be linked to different clinical outcomes. For instance, high numbers of T lymphocytes were associated with good prognosis in many human cancer types (4, 5). Also, immune modulatory chemokines were related to the natural course of cancer as well as to response to therapy (6, 7).

With introduction of high resolution gene expression arrays, it has become evident that a lot of prognostic gene signatures consist of immune markers (8–11). Particularly in breast cancer, several prognostic and predictive gene signatures reflect the individual immune response, independent from traditional markers like hormone receptor status or Ki-67 proliferation index (12–14). To systematically distinguish between T and B cell-related effects on the natural course of breast cancer, we previously showed that the humoral immune system, as summarized in a 60-gene B-cell signature, had a strong protective impact on metastasis-free survival (MFI) in node-negative breast cancer patients (15). However, analysis of a 60-gene signature by real-time PCR (RT-PCR) is costly and relatively labor intensive. To improve the clinical applicability, we studied whether the influence of the B-cell metagene on prognosis can be narrowed down to a single gene. We report that the prognostic information provided by both mRNA and protein levels of immunoglobulin κ C (IGKC) was comparable with that of the 60-gene B-cell metagene. Next, we identified the cellular source of IGKC and evaluated this host-

dependent signature in other common cancer types. Finally, to translate the findings to robust analytic tools for clinical diagnostics on routinely archived tissue, immunohistochemistry was applied.

Patients and Methods

Patients

Our analysis includes gene array data from 1,810 breast cancer patients (965 node negative, without chemotherapy and 845 with anthracycline-based chemotherapy), 1,056 non-small cell lung carcinoma (NSCLC), 513 colorectal, and 426 ovarian cancer patients. In addition, paraffin-embedded tissue blocks of 330 node-negative breast cancer patients were analyzed (detailed description, Supplementary Methods).

Gene expression analysis and immunostaining

HG-U133A arrays were used to analyze Uppsala lung cancer ($n = 196$) cohorts (Supplementary Table S1). All other gene array data are publicly available (Supplementary Methods). IGKC mRNA levels in formalin-fixed, paraffin-embedded (FFPE) tissue were quantified by quantitative RT-PCR (qRT-PCR). For both immunohistochemistry and confocal-fluorescence microscopy, antibodies against MUM1/IRF4, CD20, pan-cytokeratin, or immunoglobulin G (IgG) and IGKC were used as previously described (details, Supplementary Methods).

Statistical analysis

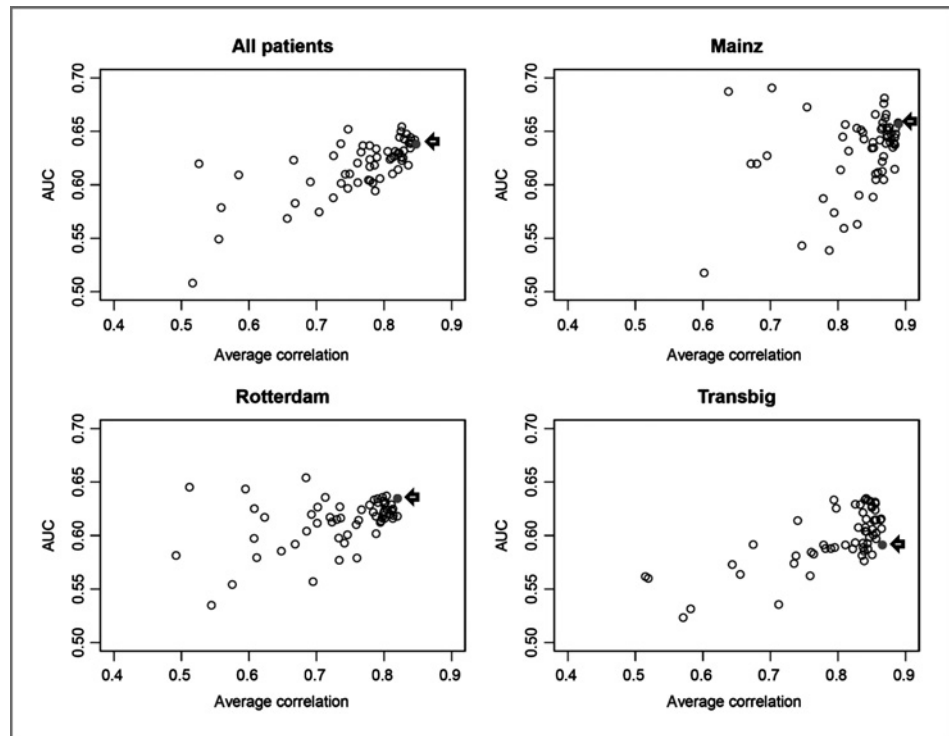
Survival was analyzed by univariate and multivariate Cox models and visualized by Kaplan–Meier plots. The Brier score was used to evaluate the ability to predict survival. Meta-analyses were conducted by fixed and random effect models and visualized with forest plots (details, Supplementary Methods).

Results

IGKC is a representative marker of the B-cell gene signature

To condense the previously described breast cancer B-cell signature (15) that consists of 60 genes, we analyzed microarray data from our own breast cancer cohort (Mainz) and 2 independent cohorts [Rotterdam (19); Transbig (16, 17)]. The bioinformatic strategy was based on the optimal combination of 2 criteria (Fig. 1): (i) the best average correlation of each of the 60 genes with all other members of the B-cell metagene as a measure of representativeness, and (ii) the largest area under the receiver operating characteristic curve, as a measure of the ability of each individual gene to discriminate between patients with and without metastasis during a 5-year follow-up period. Using these 2 criteria, IGKC was identified as one of the genes with the best average correlation and largest area under the curve (AUC; Fig. 1) and also showed a wide dynamic range with a unimodal distribution (Supplementary Fig. S1). The results obtained by microarrays were confirmed with qRT-PCR in archived FFPE tissue from the Mainz cohort. IGKC mRNA levels

Figure 1. IGKC is representative for the B-cell metagene. Each spot represents one of the 60 genes of the B-cell metagene. IGKC is indicated by the red color (and additionally by an arrow). Average correlation is the mean of all absolute Pearson correlations of an individual gene with all other members of the B-cell metagene. AUC is a measure for the ability of the corresponding gene to discriminate between patients with and without metastasis. High values indicate better prognosis.



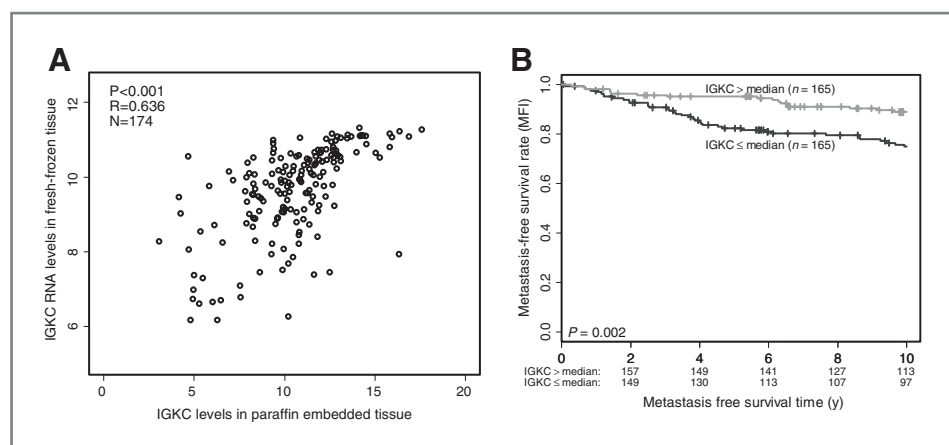
determined by qRT-PCR, correlated very well with the levels measured by gene array in fresh-frozen samples of the same tumors (Fig. 2A) and similarly, IGKC mRNA levels in paraffin-embedded tissue showed a significant association with MFI both in univariate and multivariate analyses (Table 3; Kaplan–Meier plot: Fig. 2B).

IGKC is associated with better prognosis in breast cancer

To further validate the prognostic impact of IGKC, we analyzed mRNA expression as a single marker in 5 publicly accessible gene array data sets of node-negative breast cancer patients who did not receive chemotherapy: the Mainz (15), Rotterdam (19), Transbig (16, 17), Yu (18),

and NKI (20, 21) cohorts. The meta-analysis revealed a highly significant association of IGKC RNA levels with better prognosis ($P < 0.0001$, Fig. 3). The expression of IGKC was further analyzed in the 3 molecularly and biologically different subtypes of breast cancer (14): (i) estrogen receptor (ER) status positive and HER2 status negative, (ii) ER status negative, and (iii) HER2 status positive and ER status positive or negative carcinomas. High IGKC expression correlated with good prognosis in all subgroups with a particularly strong association in the HER2-positive subgroup (Fig. 3). The univariate (Table 1) and multivariate Cox regression models (Table 2) adjusted to established clinical factors (Supplementary Fig. S2) confirmed the association of IGKC with MFI (Table 1,

Figure 2. Confirmation in paraffin embedded tissue. A, RNA levels determined by gene array in fresh-frozen tumor tissue of node-negative breast cancer patients correlate with RNA levels of the same tumors determined by qRT-PCR in FFPE tissue. B, Kaplan–Meier plot for IGKC RNA levels in paraffin-embedded tissue ($n = 330$).



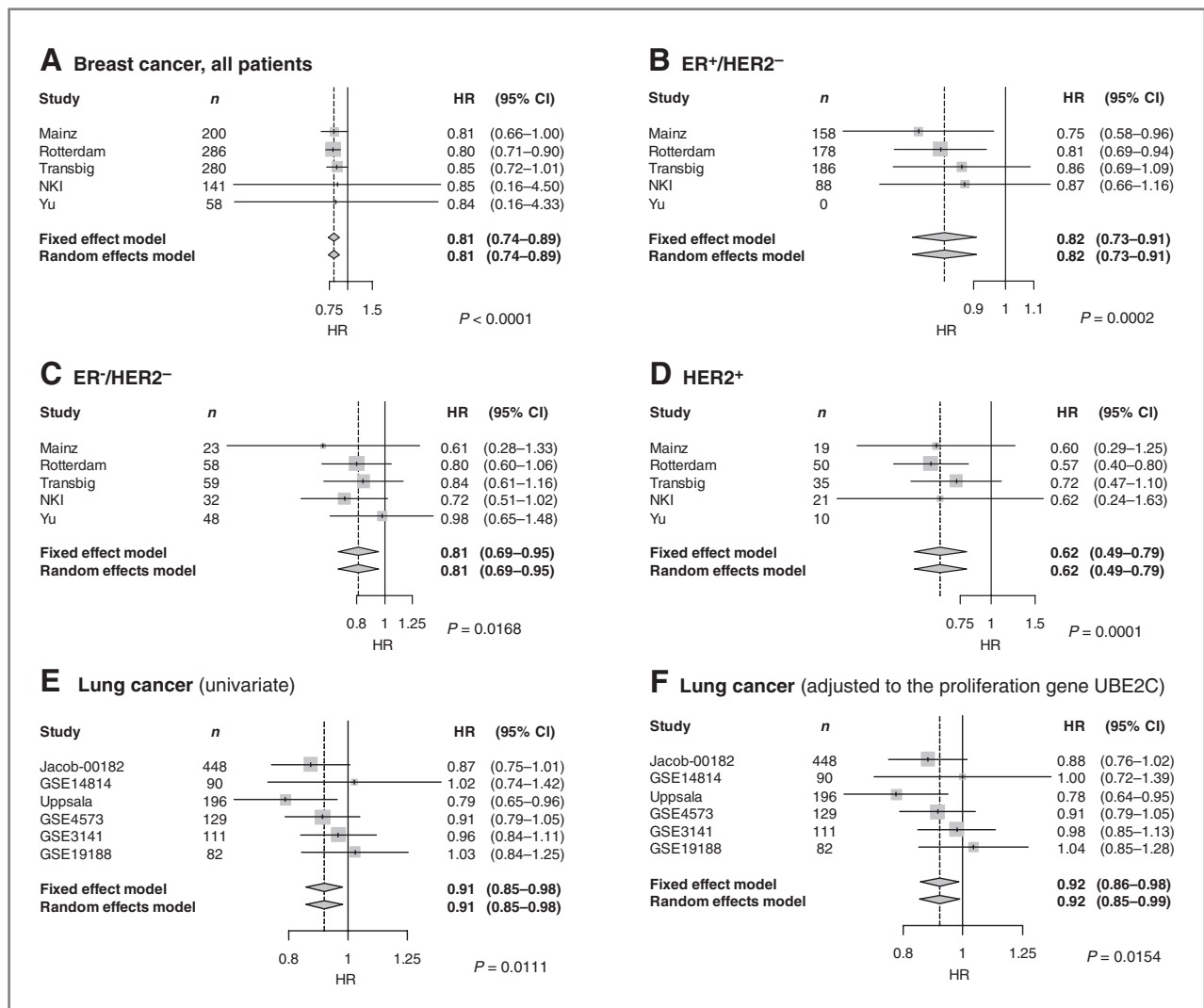


Figure 3. IGKC is associated with better prognosis in node-negative breast cancer and NSCLC. The meta-analysis shows the influence of IGKC on metastasis-free survival in 5 cohorts (Mainz, Rotterdam, Transbig, NKI, and Yu). Data are given for all patients (A) as well as for the 3 breast cancer subgroups according to Desmedt (14), namely ER positive and HER2 negative (B), ER negative and HER2 negative (C), as well as HER2 positive and ER positive or negative (D) patients. In all cohorts, high IGKC is associated with a trend toward better prognosis resulting in a highly significant association in the meta-analysis using the fixed as well as the random effects models. The Yu cohort contains only ER-negative patients and only 1 event in the HER2-positive group and was excluded from the analyses in B and D. IGKC expression is also prognostic in NSCLC either in the univariate Cox model (E) or in the model adjusted to the proliferation marker UBE2C. *n*, number of patients; *P*, *P* value of the fixed effect model.

Supplementary Table S2), disease-free survival (Supplementary Table S3A) and overall survival (OS; Supplementary Table S3B). For further illustration, IGKC gene expression was dichotomized at the median, and Kaplan–Meier curves were plotted (Supplementary Fig. S3). IGKC correlated with recently published biological signatures (15), the B-cell metagene, and to a lesser degree, with the T-cell metagene (15). In addition, there was a weak inverse correlation with the ER metagene but not with the proliferation metagene (Supplementary Fig. S4 and S5). Brier score analysis showed that IGKC alone has a similar predictive power as the complete 60-gene-based B-cell signature (Supplementary Fig. S6).

IGKC predicts response to anthracycline-based chemotherapy

In addition to the prediction of survival, IGKC expression levels were evaluated with regard to response to cytostatic drugs. We selected all published gene array data of breast cancer patients who had received anthracycline-based neoadjuvant therapy (Fig. 4). High IGKC expression was associated with complete response (CR) in a meta-analysis that included 7 cohorts ($n = 845$; $P < 0.0001$, Fig. 4). Analysis of the subgroups according to Desmedt (14) showed that IGKC is predictive for response in the ER⁻/HER2⁻ and in the HER2⁺ subgroups but not in the ER⁺/HER2⁻ subgroups. In particular, the association with CR

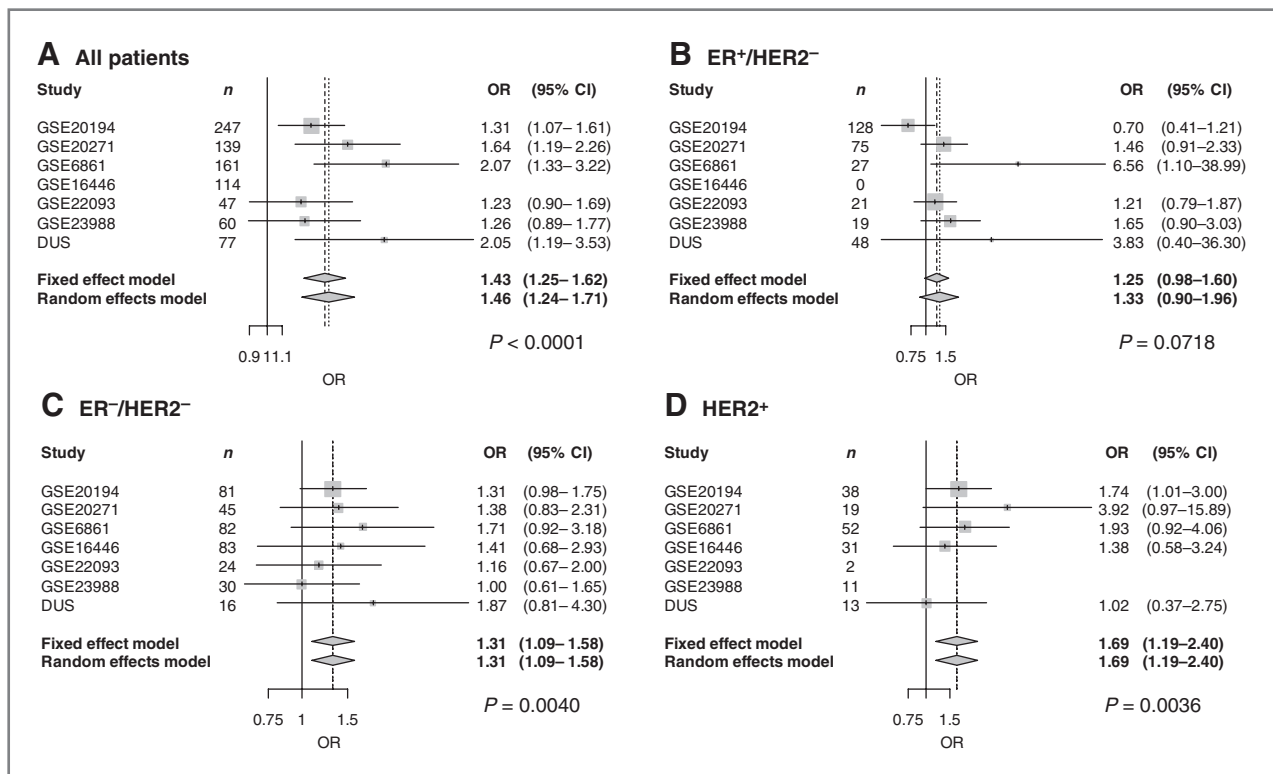


Figure 4. IGKC is associated with better response (CR) in breast cancer patients treated with anthracycline-based neoadjuvant chemotherapy. A meta-analysis of all available cohorts treated with anthracycline-based neoadjuvant chemotherapy was carried out. Data are given for all patients (A) as well as for the 3 breast cancer subgroups according to Desmedt⁴. IGKC is associated with better response to chemotherapy in the total group (A), the Desmedt subgroups shown in C and D but not in the ER-positive subgroup (B). *n*, number of patients.

was pronounced for the ER-negative patients ($P < 0.0001$, Supplementary Fig. S7A). In multivariate analyses, the association of IGKC with CR was independent of progesterone receptor (PR), HER2, proliferation status (represented by ubiquitin-conjugating enzyme E2C; UBE2C), and type of chemotherapy (anthracycline-based chemotherapy with or without additional taxane; Supplementary Fig. S7C–S7G). Again, a comparison of the ability to predict response to chemotherapy based on logistic regression models yielded similar AUC values for IGKC and the 60-gene B-cell signature (Supplementary Fig. S8). In conclusion, IGKC showed strong correlation with survival, but also predicts chemosensitivity in ER-negative patients in the neoadjuvant setting.

IGKC is also prognostic in NSCLC and colorectal cancer

Because the immune response represents a general mechanism in tumor biology, we analyzed the prognostic impact of IGKC expression in lung, colorectal, and ovarian carcinomas (Supplementary Fig. S9). For lung cancer, we evaluated a novel cohort of 196 NSCLC patients from Uppsala. Both the B-cell metagene as well as single IGKC mRNA expression were significantly associated with longer survival in the univariate ($P < 0.001$) and multivariate Cox regression model ($P = 0.032$) adjusted to established clinical factors (Supplementary Fig. S9). Interestingly, Kaplan–

Meier analysis in the subgroups revealed that this prognostic relevance was restricted to lung adenocarcinoma and was not seen in squamous lung carcinomas (Supplementary Fig. S9A and S9B), possibly because of the smaller sample size ($n = 66$). To further validate these results, we conducted a meta-analysis of publicly available Affymetrix data sets, including a total of 1,056 lung carcinomas (Fig. 3E and F). Both the univariate ($P = 0.011$; Fig. 3E) and the bivariate meta-analysis, adjusted to the proliferation marker ubiquitin-conjugating enzyme 2C UBE2C ($P = 0.015$; Fig. 3F), showed a significant association of IGKC with long-term overall survival.

Furthermore, we confirmed a significant association between IGKC and relapse-free survival in a meta-analysis of gene expression data of 513 patients with adenocarcinoma of the colorectum (Supplementary Fig. S9D). For overall survival, the association did not show significance (Supplementary Fig. S9E). No association was seen in a meta-analysis of 426 patients with ovarian cancer (Supplementary Fig. S9F).

IGKC protein expression in archived breast cancer tissue

Valuable biomarkers should be applicable for routine diagnostics. A major obstacle for gene expression studies is the limited availability of fresh tumor tissue in clinical practice. Indeed, most prognostic markers in breast cancer,

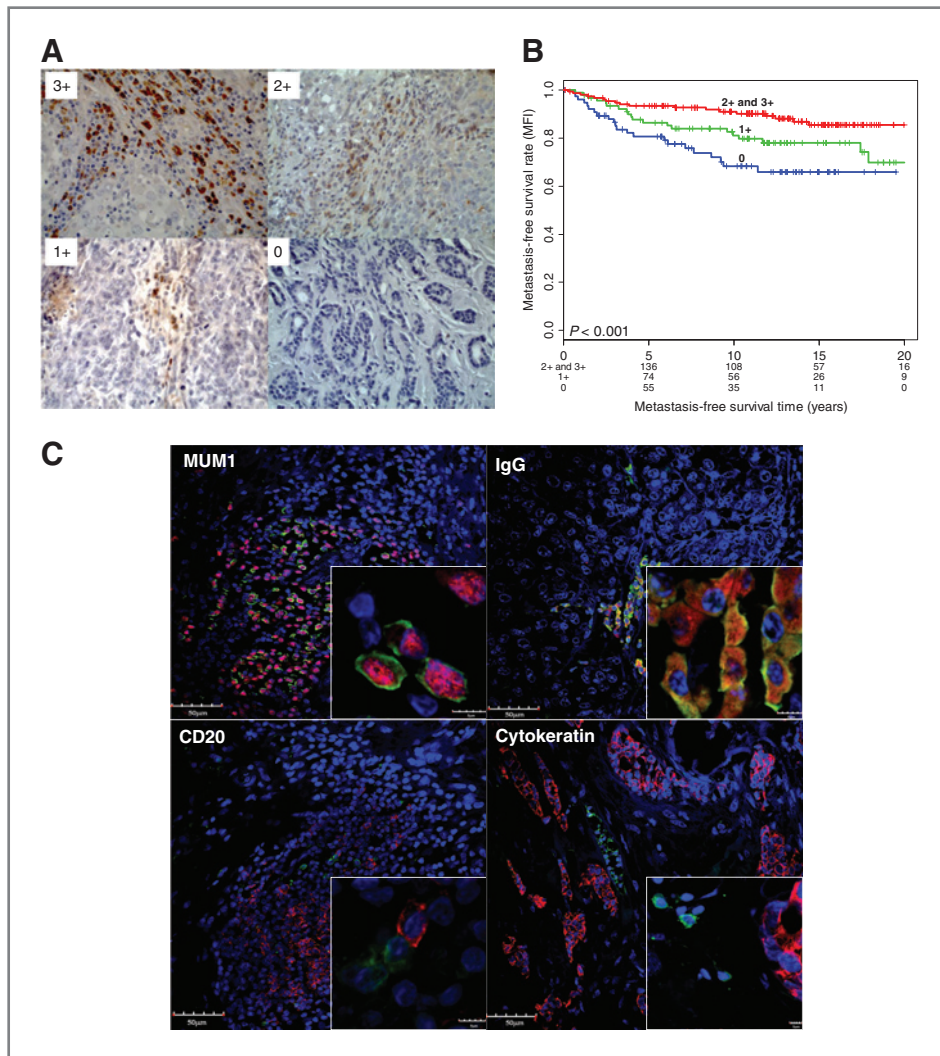


Figure 5. IGKC immunohistochemistry in paraffin-embedded tissue. **A**, IGKC immunostaining intensities 3+, 2+, 1+, and 0 in representative sections of ductal breast carcinomas; note the expression within the desmoplastic stroma in-between tumor cell nests; magnification: $\times 400$. **B**, prognostic relevance of IGKC immunostaining intensity in paraffin-embedded tumor tissue of the "Mainz node-negative paraffin cohort." **C**, coexpression by immunofluorescence of IGKC with MUM1/IRF4, a marker for plasmablasts and plasma cells; IgG coexpression illustrates the isotype switch; B-cell marker CD20; epithelial cell marker cytokeratin decorates tumor cells. IGKC is visualized by green fluorescence, whereas MUM1/IRF4, IgG, CD20, and cytokeratin emit a red signal. Scale bars, 50 and 5 μm in the overviews and close-ups, respectively. The corresponding positive and negative controls are shown in Supplementary Fig. S10.

for example, ER, PR, HER2, and Ki-67, are routinely determined by immunohistochemistry. Therefore, we tested a monoclonal antibody against IGKC in FFPE tumor samples from the Mainz breast cancer cohort and found that IGKC was expressed in lymphoid cells in the tumor stroma of breast cancer (Fig. 5A). Immunostaining intensities correlated with IGKC RNA levels isolated from the tissue slides ($P = 0.014$; Jonckhere-terpstra test comparing staining intensity groups 0 vs. 1+ vs. 2+/3+) as well as with MFI (Fig. 5B).

IGKC is expressed in tumor-infiltrating plasma cells

Finally, to identify the cell type that was responsible for IGKC expression, we carried out costaining with antibodies against IGKC and either CD20 (a B-lymphocyte marker expressed in mature B cells but not on plasma cells), pancytokeratin (a marker for epithelial cells), or MUM1/IRF4 (a marker for activated B cells, plasmablasts, and plasma cells). No colocalization between IGKC and CD20, or IGKC and cytokeratin was observed (Fig. 5C). However, more than 90% of all cells that stained positive for nuclear MUM1/

IRF4 were also positive for cytoplasmic IGKC (Fig. 5C). In addition, costaining with anti-human IgG showed that IGKC is only expressed in IgG-positive cells. Collectively, our results indicate that IGKC is expressed in mature plasma cells.

Discussion

Here, we describe a B cell-related gene signature, best represented by IGKC, as a strong prognostic marker in human breast, lung, and colorectal adenocarcinomas. Tumor-infiltrating plasma cells were identified to be the source of IGKC expression, which supports the concept that the adaptive humoral immune response is responsible for this host-dependent protective effect.

Numerous studies have shown the association of infiltrating immune cells and prognosis and response to therapy in different cancer types. However, most often the clinical relevance was ascribed to the T-cell lineage, with predominance of CD8⁺, and CD45RO⁺ T lymphocytes in colorectal, lung, and ovarian cancer (22–25).

Table 1. IGKC is associated with MFI in 3 independent cohorts of systemically untreated node-negative breast cancer (combined Mainz, Rotterdam, and Transbig cohorts, $n = 766$): univariate Cox analysis

	Mainz cohort ($n = 200$)	Rotterdam cohort ($n = 286$)	Transbig cohort ($n = 280$)	Combined cohorts ($n = 766$)
IGKC ^a				
<i>P</i>	0.052	<0.001	0.060	<0.001
HR (95% CI)	0.81 (0.65–1.00)	0.80 (0.71–0.90)	0.85 (0.72–1.01)	0.79 (0.72–0.86)

^aIGKC was analyzed as a continuous variable.

Table 2. Multivariate Cox analysis adjusted to established clinical factors (combined Mainz and Transbig cohorts, $n = 480$)

	<i>P</i>	HR (95% CI)
Age (<50 vs. ≥50 y)	0.791	1.14 (0.74–1.73)
pT stage (<2 vs. >2 cm)	0.012	1.78 (1.13–2.78)
Histologic grade (grade 1 and 2 vs. grade 3)	0.001	2.27 (1.41–3.65)
ER and PR (negative vs. positive)	0.964	1.01 (0.61–1.67)
HER2 status (negative vs. positive)	0.231	1.42 (0.79–2.53)
IGKC (continuous variable)	0.005	0.81 (0.70–0.93)

The immune infiltrates in breast cancer have been characterized recently. Our own group systematically investigates the role of B and T cells, as typified by their respective metagenes, in the natural course of medically untreated

Table 3. Prognostic relevance of IGKC determined by qRT-PCR in paraffin-embedded tumor tissue of patients ($n = 330$) with node-negative breast cancer

	<i>P</i>	HR (95% CI)
Univariate Cox analysis of MFI		
IGKC (continuous variable)	0.004	0.882 (0.809–0.960)
Multivariate Cox analysis of MFI adjusted to established clinical factors		
Age (<50 vs. >50 y)	0.307	0.944 (0.593–1.501)
pT stage (<2 vs. >2cm)	0.880	0.964 (0.601–1.547)
Histologic grade (grade 3 vs. Grades 1 and 2)	<0.001	3.853 (2.386–6.238)
ER and PR (negative vs. positive)	0.136	1.533 (0.874–2.690)
ERBB2 status (positive vs. negative)	0.405	1.277 (0.718–2.270)
IGKC (continuous variable)	0.001	0.871 (0.805–0.944)

node-negative breast cancer (15). Our description of a strong and independent prognostic impact of the humoral immune system in rapidly proliferating node-negative breast cancer was now confirmed by Bianchini and colleagues (26). Our study presents a consequent extension of the previous work with focus of B-cell lineage and a systematic implementation of solid biostatistics; therewith, we were able to condense the 60-gene B-cell signature to IGKC as a single gene. In addition to the prognostic impact, IGKC expression predicts also response to neoadjuvant chemotherapy in breast cancer. This further substantiates the hypothesis that chemotherapy does not only exert a direct cytotoxic effect but at the same time enhances the antitumor immune response (27, 28).

The robust reproduction of IGKC's clinical relevance in other cancer types represents in general one of the sparse exception that gene signatures are compatible between different cancer types. Mainly proliferation-related signatures have been shown to be transferable (29). Likewise the immunohistochemical analysis of the proliferation marker Ki-67 is of clinical importance in a variety of cancer entities. (30). In near analogy, the B-cell metagene reflects a general beneficial biological mechanism, which can easily be measured by IGKC protein staining. The validation of the gene expression findings in 330 node-negative FFPE tumors by immunohistochemistry was therefore of particular importance because fresh-frozen tissue is logistically demanding to obtain on a routine basis and often only small biopsies are available. Thus, an antibody-based detection of IGKC is applicable in routine cancer diagnostics.

Our finding that IGKC in tumors arises from plasma cells contradicts the provocative assumption that tumor cells are capable of producing immunoglobulins to promote growth and survival (31). Rather, it supports a previous report that breast cancer specimens typically have tumor infiltration of IgG-positive plasma cells (32). Similarly, another study of Wang and colleagues described that the majority of tumor-infiltrating plasma cells in invasive-ductal breast carcinomas was of IgG isotype suggesting that a tumor-derived antigen response may lead to the maturation of systemic B cells (33). In accordance, in our study, costaining for IGKC and IgG confirmed increased heavy class isotype switch to IgG. This antigen-dependent switch from immunoglobulin M and immunoglobulin D to IgG1 production is a well-known feature of B-cell maturation (34) and plasma cell

differentiation (35) after antigen encounter. Notably, several reports have characterized oligoclonal expansion of B cells in breast cancer (36–40). But none of these groups have yet shown a robust clinical impact of these intriguing findings.

Interestingly, the impact of the peritumoral immune system could be shown in other tumor entities, that is, in NSCLC and colorectal cancer, but not in ovarian cancer. We speculate that this may be explained by distinct growth pattern in different organs and subsequent different immunogenic properties. The biological roles of the IGKC signature have to be addressed in further studies. Nevertheless, the strong prognostic impact shared by breast, lung, and colorectal adenocarcinomas represents, to the best of our knowledge, the first robust comprehensive biomarker predicting the response of the immune system in a variety of cancer types.

We have to acknowledge the retrospective nature of our study, but currently prospective analyses of breast cancer without adjuvant treatment are not feasible considering current treatment recommendations (41). Also, a detailed evaluation of additional malignant tumor types is difficult because of limited clinical and pathologic data in the published expression array data sets. It should be considered that not only κ but also λ light chain-associated probe sets are among the top genes indicating an antitumor response (Supplementary Fig. S11). However, IGKC combines the advantages of not only belonging to the top genes indicating a favorable prognosis but also offers the possibility that RNA from paraffin tissue can be used, and the

results could be validated by immunostaining with commercially available antibodies.

The novelty of our study is (i) the translation of our B-cell metagene approach (15) to other tumor types, (ii) the validation by independent methods, and (iii) the establishment of IGKC as a biomarker for clinical diagnostics on FFPE tissues. In conclusion, our findings strongly support the emerging role of the immune system as a clinically relevant hallmark of cancer biology (42).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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