Can granulysin provide prognostic value in primary breast cancer?

Jelena Milovanović[1]*, Nataša Todorović-Raković[1], Tijana Vujasinović[1], John Greenman[2], Vesna Mandušić[3], Marko Radulović[1]

[1]Department of Experimental Oncology, Institute of Oncology and Radiology of Serbia, Belgrade, Serbia

[2]Department of Biomedical Sciences, University of Hull, Hull, UK

[3]Department for Radiobiology and Molecular Genetics, Institute of Nuclear Sciences Vinča - National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia

*Corresponding author:
Jelena Milovanović
Department of Experimental Oncology
Institute of Oncology and Radiology of Serbia
Pasterova 14, 11000 Belgrade, Serbia.
Tel: +381 11 20 67 289
E-mail:
jelena.mil10@gmail.com

jelena.milovanovic@ncrc.ac.rs

Abstract

Background: Granulysin (GNLY) is a cytolytic and proinflammatory molecule acting also as an immune alarmin. Its multifunctional nature has made it challenging to define its full potential as a biomarker in breast cancer.

Aim: To evaluate the relationship between intratumoral GNLY levels and clinical outcome in primary breast cancer patients.

Patients and methods: The study included 69 node-negative (N0) breast cancer patients with known clinicopathological parameters, all of whom had not received any prior hormonal or chemotherapeutic systemic therapy that could possibly interfere with the course of disease. The median follow-up period was 144 months. Intratumoral GNLY mRNA levels were determined by RT-qPCR. Prognostic performance was evaluated by the receiver operating characteristic (ROC), Cox proportional hazards regression and Kaplan-Meier analysis. Classification of patients into $GNLY^{low}$ and $GNLY^{high}$ subgroups was performed by the use of the outcome-oriented cut-off point categorization approach.

Results: There was a significant difference between GNLY values of patients without any recurrences and those with local or distant recurrences (Mann-Whitney test, p=0.05 and p=0.02, respectively). None of the tested parameters showed prognostic significance for local and distant recurrences when combined. When distant metastases and local recurrences were separated as events, the best prognostic performance was observed by GNLY as compared with clinicopathological parameters (AUC=0.24 and p=0.04 for local events; AUC=0.71 and p=0.03 for distant events). Furthermore, GNLY was the independent prognostic parameter (Multivariate Cox regression). Local recurrence incidence was 0% for the $GNLY^{high}$ subgroup and 19% for the $GNLY^{low}$ subgroup; however distant recurrence incidence was 24% for the $GNLY^{high}$ subgroup but only 3% for the $GNLY^{low}$ subgroup (Kaplan–Meier analysis).

Conclusion: High levels of granulysin prognosticate low risk of local recurrence but a high risk of distant metastasis in primary breast cancer patients.

Keywords*: granulysin; breast cancer; biomarker; prognosis.*

1. Introduction

Granulysin (GNLY), a member of the saposin-like family of proteins, is a cytolytic and proinflammatory molecule constitutively expressed by natural killer (NK) cells and after activation in CD4+ and CD8+ cytolytic T lymphocytes (Krensky and Clayberger, 2009). Granulysin is also expressed by a wide range of additional cytotoxic innate immune cells, such as NKT cells, Vδ2+ γδ T cells and CD1-restricted cells (Sparrow and Bodman-Smith, 2020). Human granulysin exists as a 15 kDa protein which is subsequently cleaved at both its amino and carboxyl termini to form a 9 kDa protein. 15-kDa and 9-kDa forms exist in different granules within an immune cell and require activation of different pathways to be released: while 15-kDa form is constitutively secreted, secretion of 9-kDa form is regulated by receptor-mediated granule exocytosis (Krensky and Clayberger, 2009).

Many studies provide evidence for the dual ability of granulysin within the immune system; in addition to killing cells directly through release of 9-kDa form, cytotoxic immune cells also release 15-kDa form to induce chemoattraction of additional immune cell populations and maturation of antigen presenting cells (APC) such as dendritic cells (Zitvogel and Kroemer 2010, Tewary et al. 2010, Castiello et al. 2011, Clayberger et al. 2012). Maturation of dendritic cells in response to infection or tumor is crucial for additional activation of cells of the adaptive immune response and therefore initiation of both arms of the immune system (Sparrow and Bodman-Smith, 2020). 15-kDa granulysin has been shown to cause differentiation of monocytes to dendritic cells, maturation of populations of immature dendritic cells and migration/chemoattraction of several immune cell populations such as NK cells, memory CD4+ and CD8+ αβ T cells, monocytes, dendritic cells (Deng et al. 2005, Sparrow and Bodman-Smith, 2020). Furthermore, both forms induce expression of a number of proinflammatory cytokines in immune cells, including regulated upon activation T cell expressed and secreted (RANTES), monocyte chemoattractant protein (MCP)-1, MCP-3, macrophage inflammatory protein (MIP)-1α, interleukin (IL)-10, IL-1, IL-6 and interferon (IFN)-α (Deng et al. 2005, Krensky and Clayberger, 2009).

Breast cancer is a complex disease and one of the most frequent malignancies in women worldwide. Metastases found in regional lymph nodes (N+ status) are the most powerful indicator of poor prognosis (Schnitt, 2001). Still, 20-30% of newly diagnosed node-negative (N0) breast cancer patients will eventually develop local or distant recurrences (McGuire and Clark, 1992). In addition, according to standard protocols, N0 breast cancer patients (the group with a favorable prognosis) will receive adjuvant systemic therapy, which

means that a large number of these patients is overtreated with no evident beneficial effect. Therefore, searching for additional prognostic biomarkers that could help distinguish N0 breast cancer patients with low- and high-risk of recurrence is of great importance. Multifunctional nature of granulysin has made it challenging to define its full potential as a biomarker, so the aim of the study was to evaluate the relationship between intratumoral granulysin levels and clinical outcome in primary N0 breast cancer patients.

2. Material and Methods

2.1 Patients

This retrospective study included 69 node-negative (N0) breast cancer patients who underwent surgical resection at the Institute of Oncology and Radiology of Serbia. Clinicopathological characteristics of the patients at the time of primary diagnosis are presented in Table 1. The report was written according to REMARK recommendations for tumor marker prognostic studies (Altman et al. 2012). Histological specimens were examined and classified according to the criteria of the American Joint Committee on Cancer / Union International Contre le Cancer (AJCC/UICC) for TNM stage, histological type and grade. Patient data were received in an anonymised form without indirect identifiers that could enable re-identification (Safe-Harbour methodology of the 2012 Health Insurance Portability and Accountability Act).

This non-interventional, retrospective study was approved by the Institutional Ethics committee of the Institute of Oncology and Radiology of Serbia and conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki), printed in the British Medical Journal (18th July 1964) and its later amendments.

This group of N0 breast cancer patients had not received any prior hormonal or chemotherapeutic systemic therapy that could possibly interfere with the course of disease. We assembled this very specific patient group from a period of over 25 years ago when low recurrence risk breast cancer patients were not prescribed systemic therapy at our hospital. This was in line with recommendations valid at that time for the lower-risk T1/2 and N0M0 patients (Rosner and Lane, 1990).

To provide insight into the prognostic performance of granulysin in breast cancer, we evaluated whether intratumoral GNLY mRNA levels were associated with the, retrospectively recorded, actual occurrence of distant and local events. Local recurrence refers to the development of locoregional changes (in the same breast or regional lymph

nodes), while distant recurrence refers to distant metastasis such as the bone, lung, liver and brain (Chen et al. 2018). The median follow-up period was 144 months.

2.2 Sample size calculation

The prospective sample size calculation was based on a pilot experiment with 30 patients. The calculation parameters for intratumoral GNLY obtained from the pilot experiment were: target power of 0.8, effect size by hazard ratio (HR) of 7, significance level of 0.05, variability in standard deviations (SD) of 0.59 and the event rate of 12%. We calculated the variability for each feature as a distance between average values of the patient subgroups with and without the actual recurrence, expressed in SD.

The required numbers were 50 patients with six events. The actual patient number was 69, with ten distant events and six local events. The actual average SD distance between the subgroups with and without recurrence was 0.64 for distant events and 0.43 for local events. The event rate was 9% for local and 14% for distant events. The effect size for intratumoral GNLY was 0.01 for local events and 8.1 for distant events. This resulted in the actual power of 0.998 for prognostication of local events and 0.986 for distant events. Calculations were performed by the two-sided stpower cox test *(Stata/MP 16 software, StataCorp, College Station, TX, USA)*.

2.3 Steroid hormone receptor (ER, PR) determination

Cytosol tumor extracts were prepared from microdisected frozen primary tumors by a following workflow: homogenization in 5 mM phosphate buffer pH 7.4-7.7 containing 20 % glycerol, 1 mM monothioglycerol and 1.5 mM EDTA, centrifugation at 800-1000 g for 30 min, followed by ultracentrifugation at 100 000 g for 60 min. The whole procedure was performed at +4°C. Tumor extract protein concentrations were assayed by the Lowry method.

Steroid hormone receptor (estrogen receptor, ER, and progesterone receptor, PR) status was determined by ligand-binding assay i.e. in cytosol tumor extracts using dextran-coated charcoal (DCC) method. This quantitative biochemical method allows for excellent quantification of the ER and PR levels and was recommended by the European Organisation for Research and Treatment of Cancer (EORTC) (1980). The intra-laboratory quality assessment of the steroid hormone receptor levels was performed periodically following the EORTC recommendation (Romain et al. 1995). The cut-off value for the qualitative classification of positive receptor status was 10 fmol per mg of cytosol protein for ER and 20

fmol per mg of cytosol protein for PR. At the time when these patients were treated, DCC was the standard method of steroid receptor determination at our hospital.

2.4 HER2 estimation

HER2 status (absence or presence of gene amplification) was determined on formalin-fixed paraffin-embedded tumor tissue sections by chromogenic *in situ* hybridization (CISH), according to the manufacturer's instructions *(Invitrogen SPOT-Light HER2 CISH Kit, USA)*. Hybridization results were evaluated in 40×, 100× and 1000× magnification. One to 5 gene copies per nucleus were defined as no amplification, while more than 6 gene copies per nucleus or large gene copy clusters in more than 50% of tumor cells defined amplification.

2.5 Real-Time PCR Assay

Samples of breast tumor tissue with an approximate volume of 2 mm^3 were homogenized on ice in the presence of ceramic microbeads for 60 seconds with an MP Fast Prep 24 homogenizer in 600 μl guanidinium thiocyanate solution (RLT buffer, *Qiagen Inc., Santa Clarita, CA*) supplemented with 0.1 M 2-mercaptoethanol. The homogenate was further processed by centrifugation for 2 min at 12 000 x g in a QIAshredder homogenizer *(Qiagen)*.

Total RNA was then isolated with the *RNeasy mini kit (Qiagen)*. The integrity of the isolated RNA was examined by the *Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA)*. Only RNA samples with integrity number (RIN) > 7 were subsequently reverse transcribed with the *High Capacity cDNA reverse transcription kit (Thermo Fisher Scientific,Waltham, MA)* by use of hexanucleotide random primers.

Quantitative PCR was performed with the Taqman Universal PCR Master Mix, *No AmpErase UNG kit* containing AmpliTaq Gold DNA polymerase *(Thermo Fisher Scientific)* and TaqMan assay Hs00247389_m1, containing the probe which spans exons 2 and 3 junction. Transcripts were amplified for 40 cycles for 15s at 95°C, and 60s at 60°C by a 7900TH TaqMan robot *(Thermo Fisher Scientific)*. 18S rRNA was used as a normalization control for mRNA input. Only samples with a Ct<15 for 18S rRNA were considered for further analysis.

2.6 Prognostic performance evaluation

The receiver operating characteristic (ROC) analysis by the area under the ROC curve (AUC) was employed as a quantitative measure of prognostic discrimination efficiency

across all possible thresholds of the continuous quantitative mRNA values. Discrimination is the capability to stratify patients who experience the event and patients who do not experience the event. AUC ranges from 0.5 (chance accuracy) to 1.0 (perfect accuracy), with the intermediate benchmarks of: 0.4-0.5 or 0.5-0.6 (poor), 0.3-0.4 or 0.6-0.7 (fair), 0.2-0.3 or 0.7-0.8 (moderate), 0.1-0.2 or 0.8-0.9 (good) and 0.0-0.1 or 0.9-1.0 (excellent) (Yang et al. 2019). Kaplan–Meier analysis was done for the period from tumor extraction surgery until the occurrence of local and distant events *(IBM SPSS Statistics for Windows version 24, IBM Corp. Chicago, IL)*. While ROC analysis was based on continuous feature values, Cox proportional hazards regression used categorized feature values.

Categorization of the measured mRNA continuous values was achieved by the outcome-oriented optimal cut-off point selection by use of the log-rank test and the X-tile 3.6.1 software from Yale University *(New Haven, CT, USA)* (Camp et al. 2004). Univariate Cox proportional hazards regression test was performed for comparison of the prognosticated and actual, local and distant events. The HR designates the effect size by Cox regression, corresponding to recurrence rates in high- and low-risk groups of patients *(IBM SPSS)*. Each feature satisfied the proportional hazards assumption based on the Schoenfeld residuals by phtest *(Stata/MP 16 package, StataCorp, College Station, TX, USA)*. Multivariate stepwise Cox proportional hazards regression analysis was performed to test for the independence of each prognostic parameter. Variables categorized by the outcome were added to a full model using the forward selection entry criterion of $p < 0.2$ in univariate analysis and removed using backward elimination by the selection stay criterion of $p \leq 0.05$ *(IBM SPSS)*.

2.7 Validation

The p-values and confidence intervals (95%CI) of the obtained HRs and AUCs were corrected for bias using the bootstrap internal validation in *IBM SPSS Statistics for Windows version 24*. Bootstrap resample validation is a very powerful tool for testing model stability by constructing confidence intervals and calculating p-values (Efron, 1979). The bootstrap variant of “resampling with replacement” produces new "surrogate" data sets with the same number of cases as the original data set. This is achieved by a random selection of observations from the original sample until the same number of observations is achieved, followed by calculation of prognostic estimates such as the 95%CI and p-value. The performed bootstrap is defined as “resampling with replacement” because the selected observations are not removed from the pool during resampling. Therefore, some measurements may be selected multiple times while certain observations may not appear in a

resample. By creating 1000 different resamples bootstrapping offers a more stable estimate of the prognostic performance.

3. Results

Table 1 presents clinicopathological characteristics of node-negative breast cancer patients at the time of primary diagnosis. Table 2 indicates the statistical analysis of the prognostic performance of clinicopathological characteristics and intratumoral granulysin. During the follow-up time, 23% of patients developed local or distant recurrence. The calculation was performed based on the following endpoints: only local, only distant, or distant+local events.

Using ROC analysis for evaluation, none of the tested parameters showed prognostic significance for local and distant events when combined (Table 2). When local and distant recurrences were separated as events, the best prognostic performance was observed by GNLY as compared with clinicopathological parameters (AUC=0.24 and p=0.04 for local events; AUC=0.71 and p=0.03 for distant events). ROC analysis of the intratumoral GNLY levels in the prognosis of distant and local recurrences is presented in Figure 1 (A and B). AUC below or above 0.5 indicates a prognostic association with good or poor disease outcome, respectively. For instance, AUC of 0.71 with distant metastases as the endpoint indicated that patients with intratumoral GNLY mRNA levels above the outcome-oriented threshold had a higher risk of incurring an event compared to patients with GNLY mRNA levels below the threshold. In contrast, AUC of 0.24 with local relapses as the endpoint indicated that patients with intratumoral GNLY mRNA levels above the outcome-oriented threshold had a lower risk of incurring an event compared to patients with GNLY mRNA levels below the threshold. The cut-off point for intratumoral GNLY was at 18.4 dCt for both distant and local recurrences.

Using univariate Cox proportional hazards regression analysis for evaluation, none of the tested parameters showed prognostic significance for local and distant events when combined (Table 2). When distant metastases and local recurrences were separated as events, the most pronounced HR by the Cox regression was observed with age, ER and GNLY for distant metastases and with age, ER, PR and GNLY for local recurrences (Table 2). HR below or above 1.0 indicates a prognostic association with good or poor disease outcome, respectively. The multivariate Cox proportional hazards regression analysis of the distant metastasis and local recurrence risk included parameters which satisfied the forward entry criterion of P <

0.2 in the univariate analysis and the backward elimination criterion of $P < 0.05$ (Table 3). GNLY has reached the most pronounced HR when considering either distant metastases or local recurrences as endpoint events (HR=0.01 and p=0.001 for local events; HR=7.5 and p=0.03 for distant events). This analysis highlighted granulysin as the independent prognostic parameter in primary breast cancer.

The average ± standard deviation (SD) dCt GNLY mRNA levels were 20.0±1.8 for patients with distant metastases, 18.3±2.3 for patients without any recurrences and 17.0±1.3 for patients with local recurrences. Mann-Whitney rank sum test didn't show a significant difference between GNLY values of patients without any recurrences and those with recurrences (local+distant). When distant metastases and local recurrences were separated as events, there was a significant difference between GNLY values of patients without any recurrences and those with local or distant recurrences (Mann-Whitney test, p=0.05 and p=0.02, respectively). As expected, there was a significant difference between GNLY values of patients with distant metastases and those with local recurrences (Mann-Whitney test, p=0.002). By Spearman's rank order correlation test, intratumoral GNLY correlated significantly with ER and tumor grade (Table 4). A positive correlation was found between ER and GNLY levels (r=0.27) and a negative correlation between tumor grade and GNLY levels (r=-0.30). Considering clinicopathological parameters, age correlated significantly (positively) with menostatus and ER; and ER correlated positively with menostatus and PR (Table 4).

Kaplan-Meier estimator plots further illustrate the prognostic association of intratumoral GNLY (Figure 1C and 1D). Classification of patients into $GNLY^{low}$ and $GNLY^{high}$ subgroups was performed by the use of the outcome-oriented cut-off point categorization approach. P-values were calculated by the Cox proportional hazards regression test. A wider separation between upper and lower curves indicates better prognostic performance. Local recurrence incidence was 0% for the $GNLY^{high}$ subgroup and 19% for the $GNLY^{low}$ subgroup; however distant recurrence incidence was 24% for the $GNLY^{high}$ subgroup but only 3% for the $GNLY^{low}$ subgroup (Figure 1C and 1D). High levels of GNLY thereby prognosticated low risk of local recurrence but a high risk of distant metastasis. These results were further supported by Long Rank test. Survival analysis for subgroups of patients formed according to the GNLY cut-off point (18.4 dCt) showed statistical significance only when distant and local recurrences were separated as events. There was a significant difference in recurrence free survival (RFS) between patients without any recurrences and those with local or distant recurrences (Long-Rank test, p=0.01 and p=0.02, respectively).

4. Discussion

The only way to perceive the real clinical course of breast cancer and the prognostic significance of potential biomarkers, is follow-up of patients that had not received any adjuvant (postoperative) therapy that could possibly interfere with the course of disease. Studies regarding this issue are scarce, since adjuvant systemic therapy is prescribed to all primary breast cancer patients nowadays. The main finding of our study, in a very specific adjuvantly untreated node negative patient group with a median follow-up of 12 years, is that raised granulysin levels prognosticated low risk of local recurrence but a high risk of distant metastasis.

Our interest in defining potential prognostic value of multifunctional granulysin in primary breast cancer was also encouraged by many studies that supported a potential prognostic and predictive value of granulysin-expressing immune cells and immune-related signatures in human cancers. Several studies showed that tumor-infiltrating lymphocytes (TILs), particularly intraepithelial CD8+ T cells, had favorable effect on patients' survival in colorectal carcinoma (Ropponen et al. 1997, Naito et al. 1998, Chiba et al. 2004). A study by Sato et al. showed that intraepithelial CD8+ T cells and a high CD8+/regulatory T cell ratio were associated with favorable prognosis in ovarian cancer (2005). Another study by Seo et al. showed that CD8+ T cells were an independent predictive factor for pathological complete response (pCR) to primary systemic therapy, irrespective of breast cancer subtype and chemotherapeutic regimen (2013), while a study by Loi et al. reported for the first time an association between higher levels of TILs and increased trastuzumab benefit in HER2+ breast cancer patients (2014). A study by Chen et al. in a patient group similar to ours, and that is untreated node negative breast cancer patient group with a long-term follow-up, showed that intratumoral CD8+ T cells were associated with favorable long-term outcome (2014). Several studies found greater TIL count and increased immune-related gene expression (e.g. a T cell metagene or a B cell/plasma cell metagene) associated with better survival of breast cancer patients, even in the absence of any systemic adjuvant therapy (Rody et al. 2009, Bianchini et al. 2010, Karn et al. 2011). Manjili et al. investigated prognostic signatures of tumor-immune interactions in breast cancer patients: whereas tumor-infiltrating dendritic cells, M1 macrophages, T helper 1 (Th1) cells, CD8+ T cells and NK cells were associated with favorable prognosis, M2 macrophages, myeloid-derived suppressor cells, T regulatory cells and Th2 cells were associated with poor prognosis (2012).

Literature related to the prognostic value of granulysin in human cancers is scarce. A study by Kishi et al. showed that impaired expression of granulysin in peripheral NK cells correlated with tumor progression (2002). Two studies found upregulated mRNA expression of intratumoral granulysin associated with a favorable outcome in patients with colorectal cancer (Pages et al. 2005, Galon et al. 2006). In the second study, patients with increased expression of genes for Th1 adaptive immunity, that included interferon regulatory factor 1, CD3, CD8, granzyme B, granulysin and interferon-γ, had the best prognosis (Galon et al. 2006). A study by Saigusa et al. showed that low preoperative GNLY levels were associated with more frequent hepatic and peritoneal metastases and with a poor outcome of gastric carcinoma patients (Saigusa et al. 2007). A recent study by Roncati et al. showed that the cytotoxicity supported by Tk TILs, and that is CD8+ killer subpopulation that secreted granulysin, perforin, granzyme B and T cell intracellular antigen 1 upon activation, was an important favorable prognostic factor in breast cancer patients (Roncati et al. 2016). In this study, a 'brisk' infiltrate (a real aggression inside neoplastic core by TILs) correlated with a survival time over 10 years (Roncati et al. 2016). In accordance with these studies, we found intratumoral granulysin to prognosticate a favorable outcome of patients with local recurrences (compared to patients without any recurrences). Although the general concept is that spread is incremental from local to distant, we found in this study intratumoral granulysin to prognosticate an unfavorable outcome of patients with distant metastases (compared to those without any recurrences). It is not unusual that multifunctional biomarker change its role during the course of disease. Our results suggest that granulysin could act in different ways in different circumstainces and contexts. It is well known that pathological interactions between cancer cells and host immune cells in the tumor microenvironment create an immunosuppressive network that promotes tumor progression. Tumor tolerance/tolerization is the result of imbalances in the tumor microenvironment, including alterations in antigen-presenting-cell subsets, co-stimulatory and co-inhibitory molecule alterations and altered ratios of effector T cells and regulatory T cells (reviewed by Zou, 2005). Two recent studies found metastatic breast cancers immunologically more inert than the corresponding primary tumors (Ogiya et al. 2016, Szekely et al. 2018). A study by Szekely et al. also observed coordinated downregulation of a broad range of chemotactic and immune activating cytokines and their receptors that further contributed to the immune-cell-depleted microenvironment in metastasis; expression of activated T-cell transcription factors as well as granzyme, granulysin, IFNγ and interferon regulated genes were also low, consistent with an inactive state of T lymphocytes in metastases (2018). According to our

results, granulysin has been shown to prognosticate a poor outcome of patients with distant metastases, which could hypothetically be explained as a consequence of coordinated disregulation of a broad range of chemotactic and immune activating cytokines including granulysin, or alterations in antigen-presenting-cell subsets that may become irresponsive to granulysin and other factors, allowing metastatic breast cancer cells to evade immune surveillance.

We found in this study intratumoral granulysin levels associated with established parameters such as ER and grade. A positive correlation between ER and granulysin levels indicates potential regulation of GNLY expression via ER, through either a ligand dependent or a ligand independent pathway, as well as a possible synergistic effect of ER and GNLY in breast cancer progression. Moreover, we found ER levels significantly increased in older patients, which is in line with the published data (Clark et al. 1984; Shek and Godolphin, 1989; Hanahan and Folkman, 1996). Estrogen levels are known to be low and constant in the group of patients older than 59 years, since they are all postmenopausal (Hanahan and Folkman, 1996). It has been hypothesized that the growth of micrometastasis in this patient group is controlled by overexpression of unliganded ER (Osborne, 1998). Furthermore, we showed in this study that higher granulysin levels correlated with well-differentiated and low-proliferative G1 tumor grade which is a marker of favourable outcome (Elston and Ellis, 1991).

Although we satisfied the sample size requirement and the patient group was highly homogenized, limitations of this study include the patient group size. Additional studies in external and larger patient groups are needed to further verify the clinical validity of the reported results.

5. Conclusion

In conclusion, our results indicate potential prognostic significance of granulysin in primary breast cancer and association with established parameters such as ER and grade. High levels of granulysin prognosticate low risk of local recurrence but a high risk of distant metastasis. This study provides the first prognostic evaluation of intratumoral granulysin in a very specific adjuvantly untreated node negative patient group with a long term follow-up. Clinical applicability of the study is based on the importance of prognosis for the identification of patients at high risk of recurrence who may benefit from more aggressive personalized treatments.

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Declaration of competing interest

The authors declare no conflict of interest.

Authors' contributions

All authors have made substantial contributions to: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) and final approval of the version to be submitted.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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7. Tables

Table 1. Clinicopathological characteristics at the time of primary diagnosis

Parameter	*Patient number*	*%*
Age (years)		
≤ 59 (median)	34	49
> 59	35	51
Recurrence		
distant	10	14
local	6	9
(distant+local)	(16)	(23)
no recurrence	53	77
Menopausal status		
premenopausal	14	20
perimenopausal	6	9
postmenopausal	49	71
Tumor size (cm)		
≤ 2 cm	48	70
> 2 cm	21	30
Histological type		
Invasive ductal	31	45
Invasive lobular	15	22
other types	23	33
Histological grade		
G1	8	12
G2	58	84
G3	3	4
Estrogen receptor status		
ER−	17	25
ER+	52	75
Progesterone receptor status		
PR−	45	65
PR+	24	35
***HER2* status**		
HER2−	32	46
HER2+	15	22
data not available	22	32

Abbreviations: ER, estrogen receptor; ER− , ER < 10 fmol/mg; ER+, ER ≥ 10 fmol/mg; PR, progesterone receptor; PR−, PR < 20 fmol/mg; PR+, PR ≥ 20 fmol/mg; *HER2*, human epidermal growth factor receptor 2; *HER2–*, HER2 gene not amplified; *HER2+*, HER2 gene amplified.

Table 2. Prognostic performance of clinicopathological parameters and granulysin

Variable	Distant metastasis		Local recurrence		Distant and local events	
	AUC[a] 95% CI[c] P-value[c]	HR[b] 95% CI[c] P-value[c]	AUC[a] 95% CI[c] P-value[c]	HR[b] 95% CI[c] P-value[c]	AUC[a] 95% CI[c] P-value[c]	HR[b] 95% CI[c] P-value[c]
Age	0.69 0.53 − 0.83 **0.05***	5.7 1.55 – 181.3 **0.01***	0.28 0.13 − 0.43 0.07	0.26 0.003 – 27.1 **0.03***	0.53 0.38 − 0.69 0.70	1.1 0.37 − 2.7 0.91
Tumor size	0.63 0.43 − 0.81 0.20	3.4 0.4 − 73.0 0.07	0.57 0.32 − 0.81 0.60	1.5 0.19 − 63.4 0.64	0.62 0.45 − 0.78 0.16	1.9 0.70 − 8.7 0.25
ER	0.58 0.34 − 0.81 0.43	4.3 1.1 − 24.3 **0.009***	0.45 0.23 − 0.68 0.70	0.03 0.02 − 0.04 **0.001***	0.53 0.35 − 0.71 0.70	2.2 0.7 − 6.1 0.11
PR	0.55 0.33 − 0.78 0.60	2.4 0.61 − 13.1 0.13	0.31 0.09 − 0.53 0.11	0.04 0.03 − 0.04 **0.001***	0.45 0.27 − 0.63 0.55	1.9 0.64 − 5.2 0.20
Histological grade	0.39 0.19 − 0.59 0.25	0.45 0.12 − 2.6 0.22	0.63 0.39 − 0.86 0.30	4.3 0.88 − 48.1 0.08	0.48 0.31 − 0.65 0.78	1.20 0.29 − 6.1 0.84
Histological type	0.61 0.36 − 0.86 0.40	1.49 0.19 − 3.2 0.22	0.60 0.18 − 1.0 0.64	1.07 0.18 − 2.7 0.90	0.62 0.39 − 0.84 0.31	1.35 0.50 − 2.5 0.31
HER2	0.53 0.31 − 0.75 0.78	1.74 0.03 − 9.6 0.45	0.51 0.26 − 0.76 0.96	1.31 0.03 − 10.3 0.77	0.53 0.35 − 0.70 0.79	1.55 0.31 − 5.3 0.43
GNLY	0.71 0.56 − 0.86 **0.03***	8.1 1.5 − 90.5 **0.04***	0.24 0.10 − 0.39 **0.04***	0.01 0.006 − 0.022 **0.001***	0.53 0.37 − 0.69 0.69	1.2 0.39 – 3.8 0.72

[a] ROC analysis prognostic test, based on continuous parameter values prior to their categorisation.
[b] Univariate Cox proportional hazards regression test, based on categorized parameter data.
[c] bootstrap corrected
* $P < 0.05$

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; *HER2*, human epidermal growth factor receptor 2; GNLY, granulysin.

Table 3. Multivariate Cox proportional hazards regression analysis of the prognostic features[a,b,c,d,e]

Parameter	P-value[e]	HR	95% CI[e]
	Distant metastases[b]		
Age	0.08	5.0	1.04 − 545795
GNLY	**0.03***	7.5	1.81 − 412503
	Local recurrences[c]		
Age	**0.001***	0.02	0.009 − 0.057
GNLY	**0.001***	0.01	0.004 − 0.032

[a] Cox multivariate stepwise regression was performed by the forward entry criterion of $P < 0.2$ and the backward elimination criterion of $P < 0.05$. Only the remaining features are thus presented in this Table.
[b] Analysis performed on the basis of distant metastases as events.
[c] Analysis performed on the basis of local recurrences as events.
[d] Performed by use of categorized data
[e] Bootstrap corrected
* $P < 0.05$ indicated statistical significance.

Abbreviations: GNLY, granulysin.

Table 4. Correlations between intratumoral GNLY mRNA levels and the major clinicopathological parameters[a]

	Age	Menost.	pT	Grade	HER2	ER	PR	GNLY
Age	1.00							
Menost.	**0.75***	1.00						
pT	0.08	-0.02	1.00					
Grade	0.05	0.04	-0.15	1.00				
HER2	0.00	-0.06	-0.09	0.24	1.00			
ER	**0.44***	**0.32***	0.07	-0.16	-0.11	1.00		
PR	0.02	0.00	0.12	-0.17	0.13	**0.37***	1.00	
GNLY	0.23	0.20	0.13	**-0.30***	-0.15	**0.27***	0.21	1.00

[a] Continuous numerical values were used for calculation of Spearman's coefficients except for menostatus and disease grade, which are inherently categorical.
* Spearman's correlation coefficients with $P < 0.05$

Abbreviations: menost, menostatus; pT, tumor size; *HER2*, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; GNLY, granulysin.

8. Figures

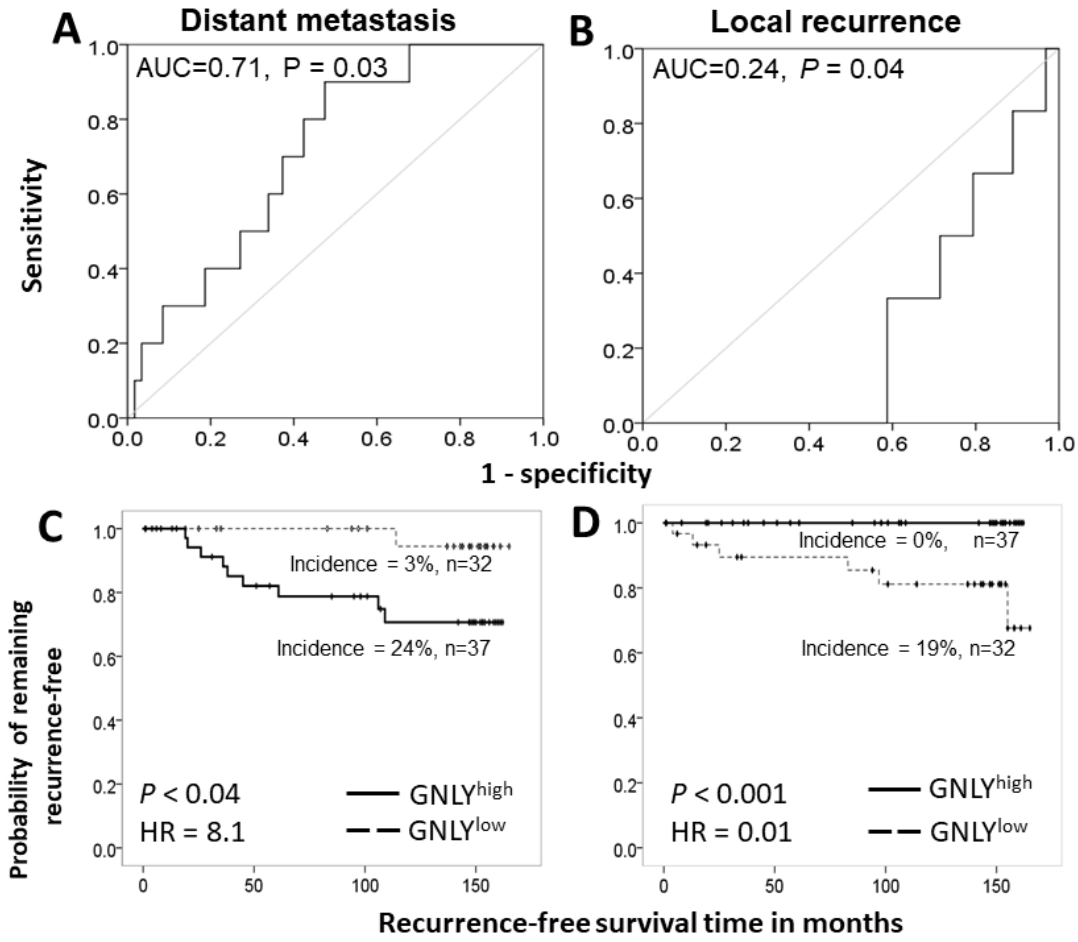

Figure 1. ROC and Kaplan Meier analysis of the intratumoral GNLY mRNA levels in prognosis of distant and local events as endpoints. (A) Prognostic performance of GNLY mRNA levels with distant metastasis as the endpoint. (B) Prognostic performance of GNLY mRNA levels with local recurrence as the endpoint. ROC analysis was based on continuous (non-categorized) feature values. (C) Kaplan-Meier prognostic analysis of GNLY mRNA levels with distant metastasis as the endpoint. (D) Kaplan-Meier prognostic analysis of GNLY mRNA levels with local recurrence as the endpoint. Classification of patients into $GNLY^{low}$ and $GNLY^{high}$ subgroups was performed by the use of the outcome-oriented cut-off point categorization approach. The cut-off point for intratumoral GNLY mRNA levels was at 18.4 dCt for both distant and local recurrences. A wider separation between upper and lower curves indicates better prognostic performance. P-values were calculated by the Cox proportional hazards regression test.

Abbreviations: ROC: Receiver operating characteristic; AUC: Area under the ROC curve; GNLY, granulysin.