Ki67 Assessment by qRT-PCR in OncotypeDx ® Breast Recurrent Score®: Low Correlation With IHC Assessment of ki67 and Prognostic Impact of ki67 RNA Level of Expression.

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Abstract

Background: Breast cancers expressing high levels of Ki67 are associated with poor outcomes. To provide individualized patient care, reliability of prognostic and predictive information deriving from Ki67 assessment is essential. Oncotype DX® test was designed for ER+/HER2- early-stage breast cancers to help adjuvant chemotherapy decision by providing a Recurrent Score® (RS®). RS® measures the expression of 21 specific genes from tumor tissue, including *Ki67*, and aim to predict the benefit of chemotherapy and the risk of distant recurrence.

The primary aim of this study was to assess the agreement between $Ki67_{RNA}$ obtained with Oncotype DX® RS® and $Ki67_{IHC}$. Other objectives were to analyze the association between the event free survival (EFS) and the expression level of $Ki67_{RNA}$; and association between RS® and $Ki67_{RNA}$.

Methods: In a cohort of 98 patients with early ER+/HER2- breast cancers, we obtained: Recurrence score®, RNA level of Ki67 expression measured when assessing RS® and Ki67 tumor labelling by immunohistochemistry ($Ki67_{IHC}$). The $Ki67_{RNA}$ was compared to the $Ki67_{IHC}$ by Kappa Cohen test. With a mean follow-up of 57 months, the association between the event free survival (EFS) and Ki67 status was measured using R package survival.

Results: This population was essentially composed of no special type tumor (91%), N0 or Nmic (71%), grade 1 (62%), and tumor size pT1c (1-2 cm) (58%). The RS® values were <18 in 38% (n=37), 18-30 in 51% (n=50) and >30 in 11% (n=11) of the patients. A low agreement of 0.24 was found by Cohen's kappa test between $Ki67_{IHC}$ and $Ki67_{RNA}$. Moreover, $Ki67_{RNA}^{high}$ tumors were significantly associated with the occurrence of events (p=0.02). On the other hand, we did not find any association between $Ki67_{IHC}$ and EFS (p=0.25).

Conclusion: We observed of low agreement between expression level of $Ki67_{RNA}$ and Ki67 protein labelling by IHC. Unlike $Ki67_{IHC}$ and independently of the RS®, $Ki67_{RNA}$ could have a prognostic value. It would be interesting to better assess the prognosis and predictive value of $Ki67_{RNA}$ measured by qRT-PCR. The $Ki67_{RNA}$ in medical routine could be a good support in countries where Oncotype DX® is not accessible.

Background

In terms of incidence, prevalence and mortality, breast cancer is in first place worldwide in women [1]. The vast majority of patients with breast cancer does not show detectable distant metastasis on diagnosis. Treatment of early breast cancer is based on surgical tumor resection with conditional lymph node dissection. Adjuvant systemic treatments including endocrine therapy (HT) and chemotherapy (CT) aim at the reduction of the distant recurrence rate and improvement of breast cancer specific survival. The decision of adjuvant therapeutic modalities is taken according to several prognostic and/or predictive

factors including patient age, tumor size, histological type and grade, lymph node involvement and expression on the tumor of hormone receptors for estrogen (ER) progesterone (PR), and the human epidermal growth factor receptor 2 (HER2) as well as the percentage of tumor cells expressing the nuclear proliferation marker Ki67 [2]. Genomic signatures were designed to give prognostic and predictive information to streamline adjuvant chemotherapy decision in ER-positive, HER2-negative breast cancer patients. Oncotype DX® Breast (ODX®) is the most widely used molecular signature in this setting and is included in treatment guidelines for estimating both the risk of distant recurrence and predicting adjuvant chemotherapy benefit. ODX® measures the RNA of 21 genes (16 cancer-associated genes and 5 housekeeping genes) and uses the expression pattern to calculate a recurrence score (RS®) that ranges between 0 and 100 [3]. The RS® result provides two types of information on tumor biology: (i) prognosis information: an estimate of the individual risk of distant cancer recurrence within 10 years, (ii) predictive information: an estimate of the likelihood of a benefit from chemotherapy [4–6].

Interestingly in breast cancer, Ki67 RNA ($Ki67_{RNA}$) is a parameter analyzed by several molecular signatures such as PAM50 and ODX [7]. Furthermore, Ki67 is an interesting biomarker in early breast cancer and breast cancers expressing high levels of Ki67 are associated with poor outcomes [8–10]. To provide individualized patient care in the concept of precision medicine, reliability of prognostic and predictive information deriving from Ki67 value is essential [11,12]. Some studies [13,14] indicate that lowering in Ki67 expression after neoadjuvant endocrine treatment may predict long-term outcome. Nevertheless, substantial variability in Ki67 staining of breast cancer tissue by immunohistochemistry (IHC) and interpretation was found between 30 routine pathology labs. Clinical use of Ki67 staining for therapeutic decisions should be considered with caution and only fully aware of lab-specific reference values [15]. Ki67 staining lacks scoring standardization ; various studies have focused on assessment methodology standardization [16], interobserver reproducibility [17] and digital image analysis of Ki67 staining [18,19]. However, little is known about variability in IHC Ki67-labelling results between routine pathology labs [20,21] and its potential influence on interpretation of Ki67 levels in breast cancer. When using Ki67 assessment by IHC in order to consider an indication of adjuvant chemotherapy [22], clinicians should be aware of the low reproducibility of Ki67 scoring and its questionable analytical validity.

In the present study, we analyzed 98 patients tested by the Oncotype DX® Breast from June 2012 to April 2014. For this cohort, $Ki67_{RNA}$ level obtained in patients' Oncotype DX® signatures were available. The primary aim of this study was to assess the agreement between $Ki67_{RNA}$ and Ki67 staining by IHC ($Ki67_{IHC}$). The other objectives were to analyze the association between the event free survival (EFS) and the expression level of $Ki67_{RNA}$ in ODX® signature; and association between RS® and $Ki67_{RNA}$.

Methods

2.1 Patients and tumors characteristics

Our study was an observational multicenter retrospective study collecting data on the real-life use of Oncotype DX Breast Recurrence Score® test by physicians in clinical practice settings in France. Patients eligibility criteria for this analysis correspond to the population for which the Oncotype DX® Breast Recurrence Score® test is validated, i.e. adult patients with a recent first diagnosis of a single early invasive breast tumor with ER+/HER2- status, plus available documentation of lymph node involvement as either N0 (node negative), Nmic (micrometastatic) or N1 (1-3 positive nodes). The following data were documented: patient age and sex, conventional clinical and pathological disease characteristics including histologic type, tumor size and grade, nodal status, receptor status including ER, PR, and HER2, and the Ki67 proliferation marker by IHC. Between 2012 and 2014, the RS® results were interpreted in three categories (low risk; intermediate risk; high risk with two cut-offs: 18 and 30). The event free survival (EFS) includes the local or metastatic relapse, other cancers and death with a mean follow-up of 57 months. Following the guidelines, the Oncotype DX® Breast Recurrence Score® test was realized after surgery and adjuvant treatments were adapted according to the RS®. The RS® values and the associated treatment (hormonal therapy plus/minus chemotherapy, or other modalities) were also collected.

2.2 Immunohistochemistry analyses.

The hormonal receptor status (ER and PR) was considered positive according to standardized European guidelines using a cut-off of ≥10% stained tumor cell nuclei. The $Ki67_{IHC}$ evaluation was realized in 2 publics and 2 private pathological departments. In these structures, the $Ki67_{IHC}$ was assessed with monoclonal antibody MIB1 [16]. Based on recommendations established by St Gallen 2015, the $Ki67_{IHC}^{high}$ corresponding to Ki67 >20%, defined “high-risk” subpopulations of tumors [23].

2.3 $Ki67_{RNA}$ threshold determination and events statistical analyses

Descriptive statistics were used to summarize clinicopathological characteristics. Variables were described by the size and rate. After normalization, levels of $Ki67_{RNA}$ were distributed according to a Gaussian curve (Supplementary data 1). We have determined the optimal threshold using R package pROC version 1.16.1 [24]. The statistical comparison between IHC and ODX® was realized by Cohen’s kappa test.

Statistical analyzes were performed using R version 3.6.2 [25]. The survival follow-up was analyzed by the package survival version 3.1-8 [26].

Results

3.1. Characteristics of the patient population (Table 1)
Complete data sets from 98 breast cancer patients who underwent RS® testing were provided from 4 public treatment centers (public hospitals and university hospitals). The patients included were exclusively female and showed a wide age distribution (31 to 81 years) with a mean age of 57 years. The predominant tumor characteristics were no special type (NST) (91%), N0 or Nmic (71%), grade 1 (62%),

and tumor size pT1c (1-2 cm) (58%). All patients had ER positive/ HER2 negative tumors. Table 1 shows the patient and disease characteristics of the full population. The RS® values were <18 in 38% (n=37), 18-30 in 51% (n=50) and >30 in 11% (n=11) of the patients. After surgery and collegial decision, all patients have received a treatment according the result of ODX® test (HT alone or CT-HT) in adjuvant situation.
During the follow-up (57 months), we observed 19 events (19%): 3 local relapses, 10 metastatic relapses (bone, lung, liver and pancreas), 3 other cancers (contralateral breast, colorectal carcinoma and pancreatic carcinoma) and 3 deaths.

Table 1. Patient and disease characteristics in the population of ER positive/HER2 negative patients.

In this population, 98 women were included from June 2012 to April 2014. All patients were tested by Oncotype DX® Breast. The Nodal status was stratified in 3 groups: N0 (node negative), Nmic (micrometastatic) or N1 (1-3 positive nodes). The progesterone receptor status (PR) was considered positive according to standardized European guidelines using a cut-off of ≥10% stained tumor cell nuclei. All patients were ER positive and HER2 negative. The $Ki67_{IHC}$ positivity was defined by the rate of >20% established by St Gallen 2015 recommendations. Almost, The Recurrence score results (RS®) are interpreted in three categories (low risk: RS® <18; intermediate risk: RS® = 18-30; high risk: RS® ≥31). The events include the local or metastatic relapse, other cancers and death.

HT: Hormonal therapy, CT: Chemotherapy, NA: not analysed , NST : no special type.

	total (n=98) (%)	
Age (years)		
< 50	34	35%
≥ 50	64	65%
Mean	57	
Tumor size (mm)		
≤10	8	8%
10 - ≤20	57	58%
20 - ≤50	32	33%
>50	1	1%
Histological type		
NST	91	93%
Other	7	7%
Tumor grade		
Grade I	61	62%
Grade II	28	29%
Grade III	9	9%
PR status		
PR <10%	18	18%
PR ≥10%	80	82%
Ki67IHC (%)		
≤20	56	57%
>20	39	40%
NA	3	3%
Nodal status		
N0	55	56%
Nmic	15	15%
N1 (1-3 nodes)	28	29%
Recurrence Score®		

Low (<18)	37	38%
Intermediate (18-30)	49	50%
High (>30)	12	12%
Treatments		
HT	72	73%
CT + HT	26	27%
Events		
No	79	81%
Yes	19	19%

3.2. Agreement between Ki67IHC and Ki67RNA

The Ki67IHC positivity rate of >20% [23] was used to define for the “high-risk” tumor group. We showed by ROC curve analysis an optimal threshold at 6.35 with a specificity of 48% and a sensitivity of 84%. With this cut off, the Ki67RNAhigh were >6.35 and Ki67RNAlow were <6.35 (Figure1). A correlation between Ki67RNA expression and Ki67IHC score was found (R=0,36; p=3.10-4 IC 95% [0.2-0.5]) (Supplementary data 2). In the cohort, 29 tumors were Ki67high and 29 were Ki67low by the two methods, which represent a match between the results in 58 patients (61%) (Table 2). Discordances were observed in 37 patients (39%): 10 samples were Ki67IHClow/Ki67RNAhigh and 27 samples were Ki67IHChigh/Ki67RNAlow. An agreement of 0.24 was found by Cohen’s kappa test.

Table 2. Agreement between Ki67RNA and Ki67IHC (n=95).

	$Ki67_{RNA}^{High}$	$Ki67_{RNA}^{Low}$
$Ki67_{IHC}^{High}$	29 (30.5%)	27 (28%)
$Ki67_{IHC}^{Low}$	10 (11%)	29 (30.5%)

Agreement of 0.24 by Cohen’s kappa test

3.3. Events-Free Survival among all patients according RS® groups and Ki67 status

A significant relationship was observed between EFS and the level of Ki67RNA expression (p=4.10-2) (Figure 2A) unlike Ki67IHC (p=0.25) (Figure 2B). However, we did not observe any significant difference on EFS according to the three RS® groups risk (p=0.4) (Figure 3).

Using the age categories from the TAILORx study [27], the association between EFS and Ki67RNA status, was found only for women under 50 years (p=0.01) and not for women over 50 years (p=0.36) (Supplementary data 3). No association between Ki67IHC and EFS was observed (p>0.20).

3.4. Ki67RNA association with RS®

As expected, Ki67RNA levels were significantly associated with RS® (p=5.10-4) (Figure 4A). To a lesser extent, an association between ODX® test with Ki67IHC status has also been observed (p=0.013) (Figure 4B). Supplementary data 4 shows the distribution of Ki67IHC score and Ki67RNA level according to patient age, tumor grade and size, PR status, nodal status, Recurrence score, treatment and events.

Discussion

4.1. Study population

In this study, we focused on the Ki67RNA expression level of 98 patients. The majority of our data are consistent with the PONDx real life study [28] which found a female population over 50 (70%) with NST tumors (78%) ranging between 2-5 cm (89%), grade II (68%) and without lymph node involvement in 79% of cases. The PONDx cohort included more grade II than in our population (68% vs 29%) with a higher contingent of lobular carcinoma (13% vs 7%). In PONDx, RS® results by prognostic category used between 2012-2014 were: <18: 54%, 18-30: 36%; >30: 10%. Compared to this real-life study, the local cohort shows an inversion of the proportions between populations at low risk (38% vs 54%) and those at intermediate risk (51% vs 36%).

The few differences observed in the distribution of the risk groups established by the ODX® test can be explained by an over-representation of grade II tumors in the PONDx study. Our cohort is comparable to this largest population for most epidemiologic parameters.

4.2. Differences between Ki67RNA and Ki67IHC

Comparisons of Ki67 status showed a discrepancy between the two different types of evaluation. Indeed, half of Ki67RNAhigh (> 6.35) were found Ki67IHClow (Table 2). The observed correlation between the two techniques, is therefore weak even if significant (R=0.36, p=3.10-4; IC 95% [0.2-0.5]) (Supplementary data2). Technically, IHC shows greater variability [20,21] and is less reproducible than the quantification of mRNAs. Currently, there is no standardization for the interpretation of the Ki67 status by IHC [16]. As a consequence, it generates inter-site and inter-observer variability within the same site [17]. In contrast, the Oncotype test which is a molecular based and standardized test provides objective results that are independent of the observer.

Furthermore, the ODX test analyzes the level of Ki67 messenger RNAs in the tumor sample. This analysis therefore considers the transcriptional status of the Ki67 gene level not only in tumor cells but also in associated cells such as lymphocytes, stromal or endothelial cells. Therefore, it is an overall estimate. By IHC, the protein expression is scored only on tumor cells. The two methods do not provide the same types of information on Ki67 status. These two types of evaluation of Ki67 are probably complementary because the Ki67IHC informs us about the tumor specific proliferation index and the Ki67RNA would reflect the proliferation of the tumor and its microenvironment. For all the reasons mentioned above, the association between RS® and Ki67IHC status is lower but remains significant (p=0.013).

4.3. Events free survival analyses

On the one hand in this study, adaptation of treatment based on RS® permit to obtain equivalent EFS in

populations with low and high risk of recurrence. We have modestly confirmed the efficiency of ODX® test. Indeed, the lack of association between RS® and EFS can be explained by the treatment (HT alone or CT-HT) performed according to the score. The clinical validity and utility of the RS® has been demonstrated prospectively across multiple studies in breast cancer patients worldwide including multiple validation studies as well as long-term prospective studies (TAILORx [27], WSG Plan B [29] and analyses from a prospective epidemiological database [30]). Based on level Ia evidence, the Oncotype DX test has been incorporated in leading internationally-accepted clinical guidelines on the treatment of early breast cancer (St. Gallen [31], ESMO [32], and ASCO [33]). This therapeutic adaptation will therefore smooth the differences between the categories of the ODX® test and at the same time demonstrates the value of the information provided by its score.
On the other hand, we were able to show that Ki67RNAhigh was significantly associated with the occurrence of events (p=0.02) but we did not find any association between Ki67IHC and EFS (p=0.25). Our data relate to a small population but leaves the possibility of a larger study in order to confirm these that Ki67RNA evaluation has a prognostic impact as RS®.

4.4. Correlation between Ki67RNA and RS®
As expected in our cohort, the RS® obtained is strongly associated with the Ki67RNA (p=5.10-4) which is one of the components of this test (Figure 4A). To optimize RS® interpretation, the TAILORx subgroup study shows a benefit of CT in women ≤50 years old with an RS® of 16 to 25 (p=0.004). CT could be avoided in women over 50 years old with a RS® <26, in women 50 years old or less with an RS® <16 [34]. Recently in San Antonio Breast cancer symposium, the results of RxPONDER study were presented [35]. And now, postmenopausal women with 1-3 positive nodes and RS® 0-25 can safetly forego adjuvant CT without compromising invasive disease-free survival. The premenopausal women with positive nodes and RS 0-25 likely significantly benefit from chemotherapy [35].
Oncotype DX® Breast enables relevant net reductions in chemotherapy use, sparing patients from serious toxicities [7]. On this other side, its clinical impact and pharmacoeconomic benefit in routine care have been shown in 20 decision-impact studies [36–38]. However, in many places, the oncotype score remains inaccessible or not reimbursed [7,28,38]. This is why the evaluation of the level of Ki67RNA by a molecular biology techniques [39,40] could be a low-cost prognostic alternative in some countries. But this hypothesis will have to be validated during a prospective translational study.

Conclusions

We observed of low agreement between $Ki67_{RNA}$ tumor level measured by qRT-PCR and Ki67 protein labelling by IHC. Substantial variability in $Ki67_{IHC}$ of breast cancer tissue and interpretation have been shown and widely published. The clinical use of Ki67 labelling should be cautious and limited by the low reproducibility of Ki67 scoring and its questionable analytical validity.

Unlike $Ki67_{IHC}$ and independently of the RS®, $Ki67_{RNA}$ could have a prognostic value. The assessment of $Ki67_{RNA}$ by qRT-PCR on breast cancer tumor would be feasible and cost effective. It would be of great

clinical utility to better assess the prognosis and predictive value of $Ki67_{RNA}$. Presentation of Ki67 status in Genomic Health report could also be helpful for therapeutic decisions on borderline situation.

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the national research committee and with the 1964 Helsinki Declaration and its later amendments. In France, this search is considered like a non-interventional study according to European legislation. All patients were individually informed that their data should be used to scientific research.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during this study (birthdate, admission date, discharge date, date of death…), are available from the corresponding author on reasonable request.

Competing interests

ZS: Honoraria from Pfizer, Genomic Health

EC: Honoraria from Novartis, Pfizer, Daiichi, Genomic Health

FB: Honoraria from Roche, Novartis, Pfizer, Astra-Zeneca, Clovis, Daiichi.

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Authors'contributions

Conception and design: EC, ZS.

Development of methodology: ZS, EC.

Acquisition of data : EC, ZS, FB, ED, MN, NM, MJP, GM, JV, XP.

Analysis and interpretation of data: ZS, AO, CM, JLP, JPF.

***Writing, review, and/or revision of the manuscript*:** ZS, CM, JLP, EC.

Administrative, technical, or material support: EC, ZS, JLP, MPA, JPF, CB.

Study supervision: EC, ZS.

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Figures

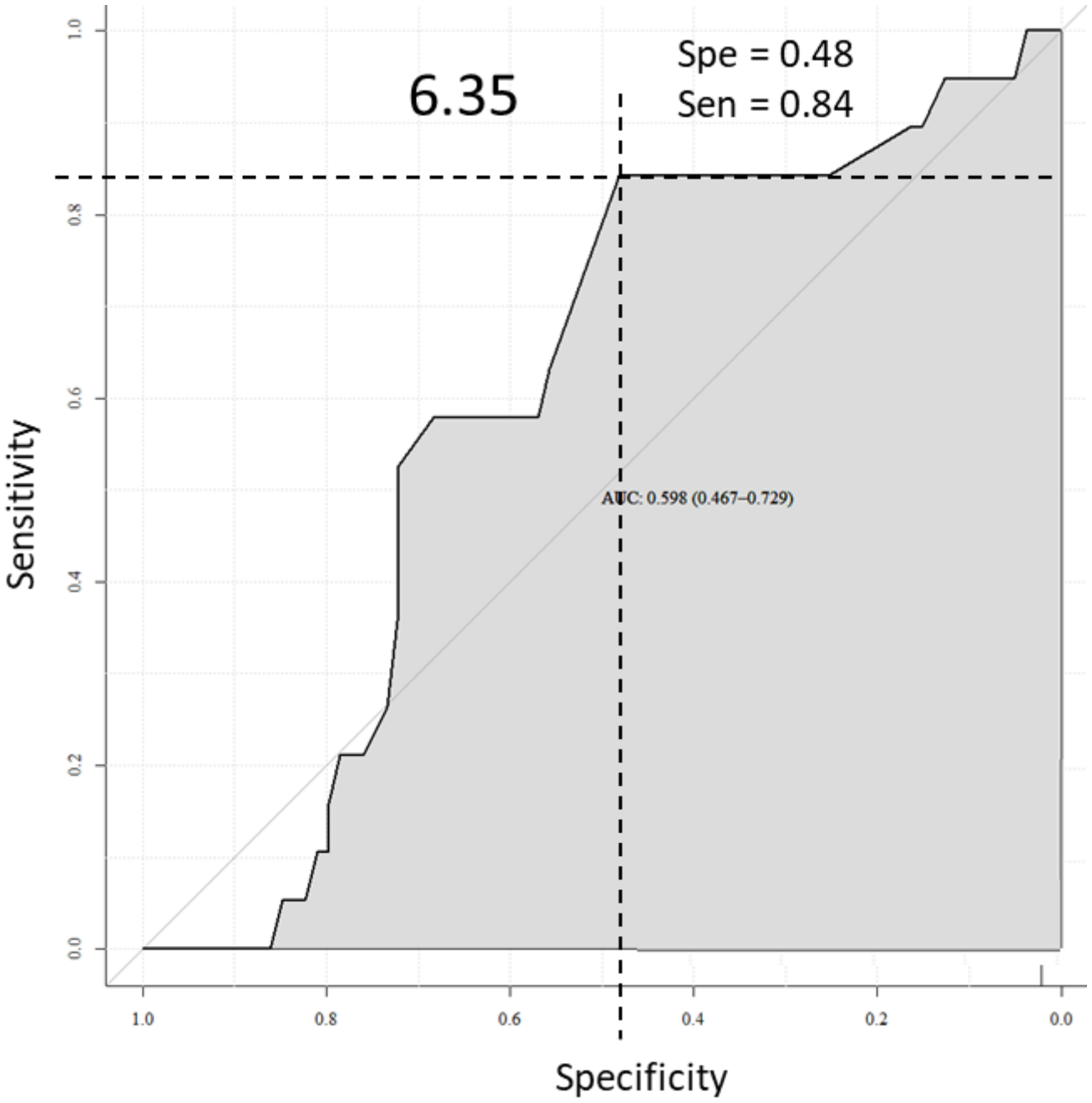

Figure 1

Threshold determination, specificity and sensitivity of Ki67RNA. Threshold of Ki67RNA was determined using package pROC version 1.16.1 (pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics, 12, p.77. DOI: 10.1186/1471-2105-12-77). An optimal threshold of Ki67RNA at 6.35 units was found with a specificity of 48% and sensitivity of 84%.

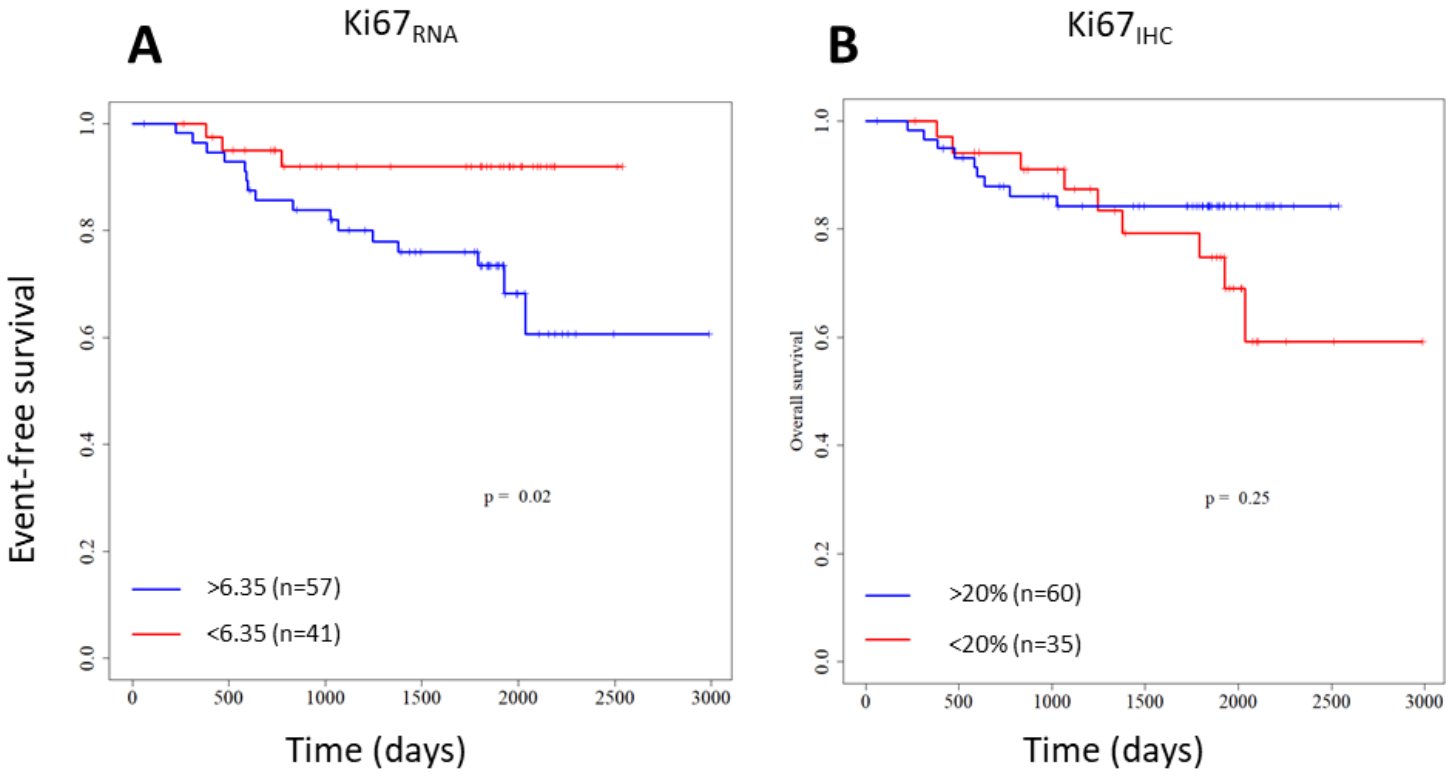

Figure 2

Events-Free Survival according Ki67 status by ODX® test and by immunochemistry. The survival follow-up was analyzing by the package survival version 3.1-8 (26) . The events include the local or metastatic relapse, other cancers occurrence and death with a mean follow-up of 57 months. (A) Ki67RNA were analyzed according to two groups using the 6.35 units' threshold. (B) The Ki67IHC were analyzed according to two groups using the rate of >20%.

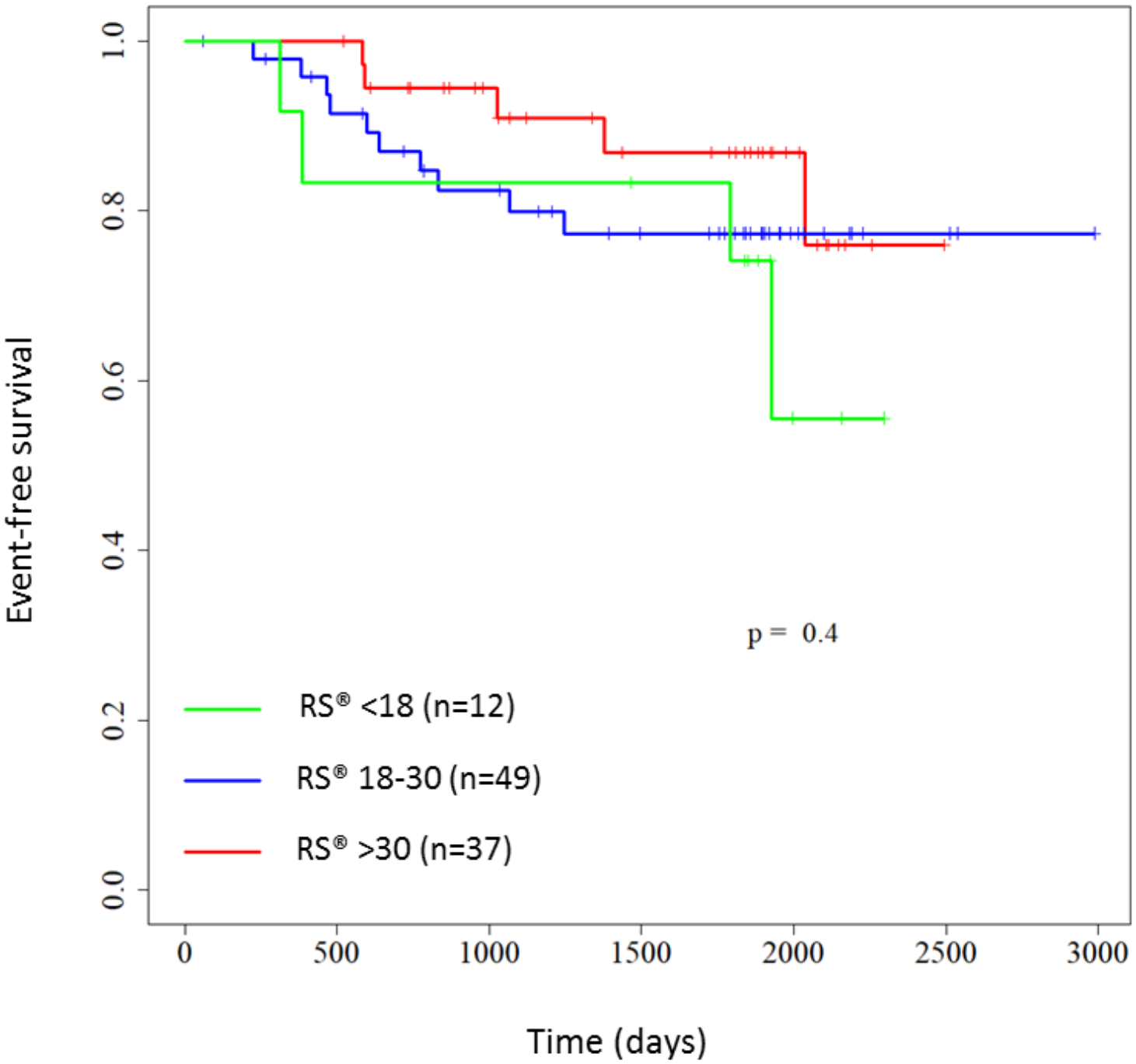

Figure 3

Events-Free Survival among all patients according RS® groups. The survival follow-up was analyzed by the package survival version 3.1-8 [26]. The events include the local or metastatic relapse, other cancers occurred and death with a mean follow-up of 57 months. The RS® are interpreted in three categories (low risk: RS® <18; intermediate risk: RS®= 18-30; high risk: RS® >30).

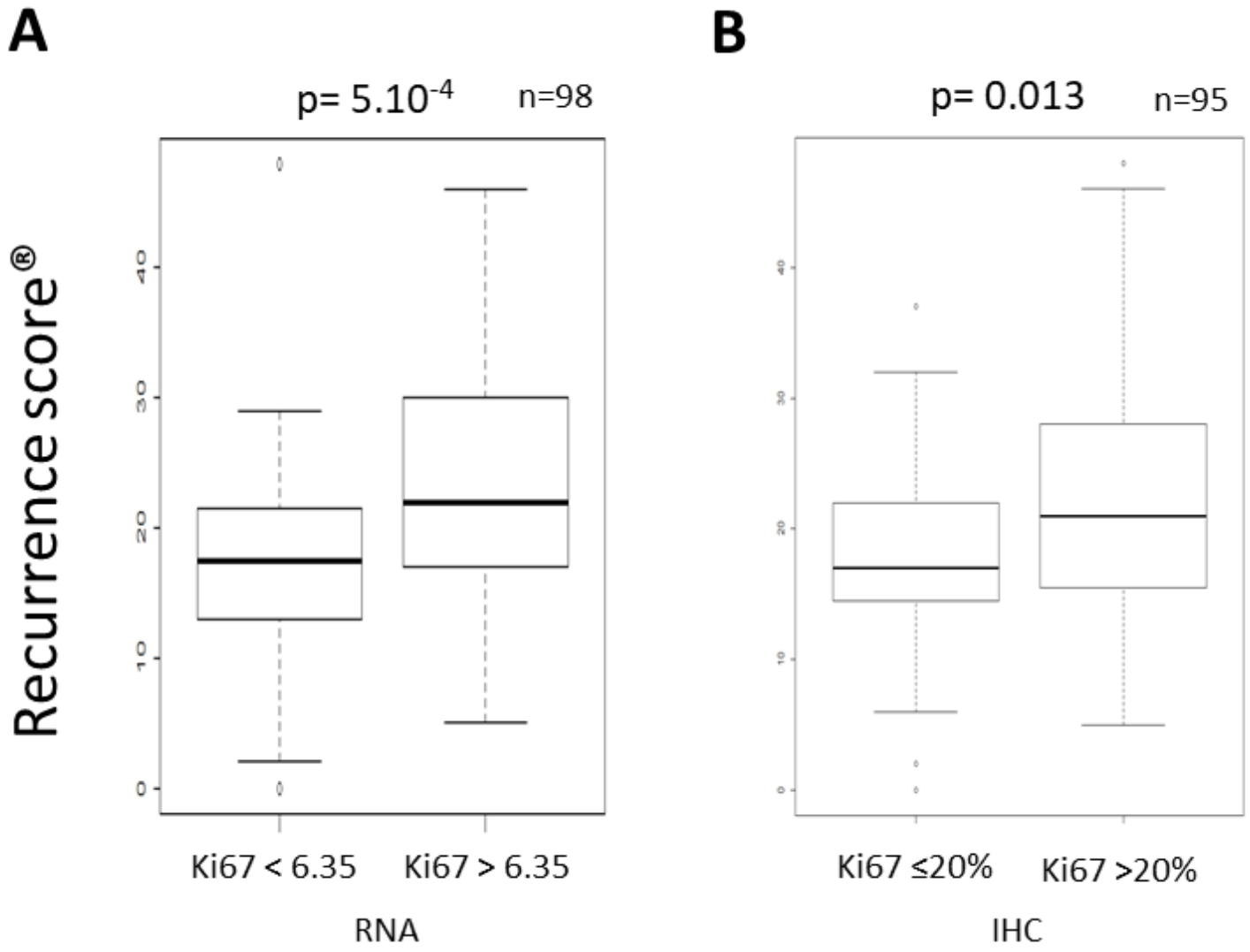

Figure 4

Association between RS® and Ki67 status A. The association of RS® with Ki67RNA were analyzed according to the 6.35 units' threshold. B. The association of RS® with Ki67IHC were analyzed using the rate of >20%.

Supplementary Files

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