RINF (CXXC5) is overexpressed in solid tumors and is an unfavorable prognostic factor in breast cancer†

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Received 21 August 2010; revised 14 November 2010; accepted 19 November 2010

Background: We have previously described the essential role of the retinoid-inducible nuclear factor (RINF) during differentiation of hematopoietic cells and suggested its putative involvement in myeloid leukemia and preleukemia. Here, we have investigated whether this gene could have a deregulated expression in malignant tissues compared with their normal tissues of origin and if this potential deregulation could be associated with important clinicopathological parameters.

Patients and methods: RINF messenger RNA expression was examined in biopsies from locally advanced breast tumors, metastatic malignant melanomas, and papillary thyroid carcinomas and compared with their paired or nonpaired normal reference samples. Further, the prognostic role of RINF expression was evaluated in locally advanced breast cancer.

Results: RINF expression was significantly higher in all tumor forms (primary breast, and thyroid cancers and metastatic melanomas) as compared with normal control tissues (P < 0.001 for each comparison). Importantly, high levels of RINF expression correlated to a poor overall survival in breast cancer (P = 0.013). This finding was confirmed in three independent public microarray datasets (P = 0.043, n = 234; P = 0.016, n = 69; P = 0.001, n = 196) and was independent of tamoxifen therapy. Notably, high levels of RINF was strongly associated with TP53 wild-type status (P = 0.002) possibly indicating that high levels of RINF could substitute for TP53 mutations as an oncogenic mechanism during the malignant development of some cases of breast cancer.

Conclusions: Our data indicate that (i) RINF overexpression is associated with the malignant phenotype in solid tumors and (ii) RINF overexpression represents an independent molecular marker for poor prognosis in breast tumors.

Key words: breast cancer, CXXC5, prognosis, RINF, tumor

Introduction

Identification of genes specifically deregulated in cancer cells could bring to light new oncogenes or tumor suppressors with clinical relevance in diagnosis, prognosis, and therapeutics. By a microarray approach, we have recently identified a novel retinoid-responsive gene (CXXC5) encoding a retinoid-inducible nuclear factor (RINF) that plays an essential role during in vitro human hematopoiesis [1]. Indeed, expression studies and gene silencing experiments both demonstrate RINF requirement not only during in vitro terminal differentiation of myeloid leukemia cells (NB4, HL60) but also during normal myelopoiesis of bone marrow progenitors (CD34+ cells in the presence of cytokines). In keeping with its essential role during myeloid differentiation and its localization to chromosome 5q31.3, a region often deleted in myeloid leukemia (acute myeloid leukemia) and preleukemia (myelodysplasia), we recently suggested RINF as a potential tumor suppressor in myeloid malignancies [1].

Even if we suspect that RINF might act as a cofactor of transcription, the molecular mechanisms by which this nuclear factor functions remain to be established. The only conserved domain of RINF is the CXXC-type zinc finger domain, a rare motif found in a small subset of proteins involved in cancer genetics, epigenetics, and chromatin remodeling through their histone methyltransferase (MLL, MLL2), histone demethylase (FBXL-10, -11, -19), DNA methyltransferase (DNMT1), or CpG-binding activities (CGBP, MBD1). In most of these proteins for which the CXXC motif has been investigated, it seems that the conserved zinc finger domain plays an essential
functional role and provides the capacity to bind unmethylated CpG [2–7], suggesting a similar role for RINF in the regulation of epigenetics. We have also described a potential nuclear localization signal, the ‘KKRRKR’-motif at amino acid residues 257–262, that recently was validated experimentally by an independent group [8].

Because RINF expression is not restricted to the hematopoietic tissue but also expressed in tissues of various embryonic origins, RINF may be involved in development and/or homeostasis of normal and pathological tissues [1, 9]. In the present study, we have investigated whether deregulations or mutations of this gene could be noticed in solid tumors derived from different tissues like the thyroid, breast gland, and cutaneous melanocytes. To our knowledge, the present report constitutes the first expression study concerning this gene that has so far escaped any clinical investigation in malignant and nonmalignant pathologies.

materials and methods

patient cohorts

We analyzed a subset of 57 of 112 patients with locally advanced primary breast carcinomas enrolled in a prospective single institution study [11]. For the present analysis, 40 patients were included in addition to benign nvi from eight healthy volunteers. Most of the biopsies were obtained from subcutaneous metastases, but there were also biopsies from lymph nodes (supplemental Table 2, available at Annals of Oncology online). Patients received standard treatment with dacarbazine monotherapy. The main characteristics of the patients in this cohort are presented in supplemental Table S3 (available at Annals of Oncology online).

RINF expression in non-tumorous breast tissue was analyzed in biopsies drawn from a pool of anonymous healthy controls with median and range of age mirroring the breast cancer patients analyzed in this study.

Malignant melanoma patients (n = 40) with distant metastases were enrolled in a prospective single institution study [11]. For the present analysis, 40 patients were included in addition to benign nvi from eight healthy volunteers. Most of the biopsies were obtained from subcutaneous metastases, but there were also biopsies from lymph nodes (supplemental Table 2, available at Annals of Oncology online). Patients received standard treatment with dacarbazine monotherapy, 800–1000 mg/m2 every 21 days.

Comparisons of the RINF mRNA expression levels in tumor and nontumor samples were carried out using the Mann–Whitney rank test (for independent samples) and Wilcoxon rank test (for dependent samples) by using the statistical software package SPSS 15.0 (IBM, Somers, NY) and/or the statistical software package SPSS 15.0 (IBM, Somers, NY) and/or the Kaleidagraph 4.1 (Synergy Software, Essex Junction, VT) software. Survival data (Kaplan–Meier) were analyzed using log-rank test.

amplification and sequencing of RINF gene

Amplification of the coding region of RINF was carried out using forward primer 5′-gggcaccttcgagcttg-3′ and reverse primer 5′-cacagacgctgctgctg-3′. PCR amplification was carried out using Dynazyme EXT DNA polymerase (FINNZYMES, Espoo, Finland) in a 50 μl reaction mixture containing 1 × PCR buffer, 1.5 mM MgCl2, 0.5 mM of each deoxynucleotide triphosphate, 5% dimethyl sulfoxide, and 0.2 μM of each primer and DNA template (0.5 μl cDNA or 1 μl genomic DNA). The PCR conditions were an initial denaturation step of 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C, followed by a final elongation step of 7 min at 72°C. After sequencing, PCR products were purified using the ExoSAP-IT kit (GE healthcare, Waukesha, WI, Cat. n° 78201). Sequencing was done using BigDye® v3.1 cycle sequencing kit (ABI, Foster City, CA, Cat. n° 4337456) with specific forward (5′-gcaaaagttgctgcctgtg-3′) or reverse (5′-gcgtggtgcaggagcat-3′) sequencing primers in a total volume of 10 μl. Thermal conditions were 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and elongation at 60°C for 4 min. Capillary electrophoresis, data collection, and sequence analysis were carried out on an automated DNA sequencer (ABI 3700).

statistical analyses

Comparisons of the RINF mRNA expression levels in tumor and nontumor samples were carried out using the Mann–Whitney rank test (for independent samples) and Wilcoxon rank test (for dependent samples) by using the statistical software package SPSS 15.0 (IBM, Somers, NY) and/or the Kaleidagraph 4.1 (Synergy Software, Essex Junction, VT) software. Survival data (Kaplan–Meier) were analyzed using log-rank test.

analysis of independent microarray

The independent microarray datasets (Affymetrix (Santa Clara, CA) GeneChip Human Genome HG-U133B and PLUS2), carried out by Miller et al. (n = 251 patient samples) [12] and Loi et al. (n = 327 patient samples) [13], were downloaded from the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE3494 and GSE6532, respectively. For GSE3494, as described in the original study, survival times were not available for 15 patients and two were censored (hence, n = 234 after filtering). For the microarray datasets with accession number GSE6532, the different patient datasets were from different institutions, the John Radcliffe Hospital in Oxford, UK (datasets OXF and OXFT), the Guys Hospital in London, UK (dataset GUYT), and the Uppsala University Hospital, Uppsala, Sweden (datasets KIT and KIU), as using specific hydrolysis probes targeting RINF gene on a LightCycler 480 machine (Roche) in accordance with the manufacturer’s instructions of the kit LightCycler® 480 ProbesMaster (Cat. N° 04 707 494 001). Relative messenger RNA (mRNA) expressions were normalized to ribosomal protein P2 (RPLP2) gene expression in a two-color duplex reaction. (Primers and thermocycling conditions are available upon request.)

RINF gene copy number analyses

Copy number analyses were carried out on genomic DNA (gDNA) by qPCRs of the RINF locus in duplex reactions with an internal reference (Beta-2-microglobulin). (Primers and thermocycling conditions are available upon request.) The qPCRs were carried out using specific hydrolysis probes targeting RINF gene on a LightCycler 480 machine (Roche) in accordance with the manufacturer’s instructions for the LightCycler® 480 ProbesMaster kit (Cat. N° 04 707 494 001). Data obtained using the RINF-specific reactions were normalized by Beta-2-microglobulin levels. These normalized values were divided by the corresponding values from a reference sample (pooled DNA form 10 healthy donors). Samples were considered to have reduced copy number if the sample/reference ratio was <0.75, and increased copy number if the ratio was >1.25.
RINF expression is increased in breast tumors and melanomas

In order to address the potential altered expression levels of RINF gene in different solid tumors, mRNA was quantified by qPCR in both tumor and nontumor tissues from patients with breast cancer, malignant melanoma, or papillary thyroid cancer. Patient characteristics are summarized in supplemental Tables 1–3, respectively (available at Annals of Oncology online), and RINF expression levels are illustrated in Figure 1. In breast cancer tissue (n = 57), the RINF expression level, relative to RPLP2 (mean 0.155, median 0.110, and range 0.025–0.576) was found to be, on average, 5.43-fold increased compared with the level measured in nontumor breast tissue (n = 7; mean 0.028, median 0.026, and range 0.021–0.043). This represents a significantly different expression level between the two groups of breast samples (P < 0.001; Mann–Whitney rank test), strongly indicating RINF to be overexpressed in breast tumors.

We further validated these findings (supplemental Figure 1, available at Annals of Oncology online) by extracting RINF data from an available independent microarray dataset [14]. Here, expression data were available for tissue within the tumors and from biopsies taken at increasing distance from the tumor. In this dataset, we also found RINF expression to be elevated in tumors compared with healthy tissue and tissue in close proximity of the tumor to express intermediate levels of RINF.

Benign nevi versus malignant melanoma displayed a similar RINF deregulated expression pattern. Indeed, in malignant melanomas (n = 40), the expression level was on average, 5.25-fold higher (median 0.089, mean 0.126, and range 0.025–0.486) to the level detected in nonmalignant nevi (n = 8; median 0.017, mean 0.017, and range 0.010–0.027), indicating RINF to be significantly overexpressed in malignant melanomas as compared with benign nevi (P < 0.001; Mann–Whitney rank test).

RINF expression is increased in thyroid tumors versus benign paired samples

The statistically significant RINF overexpression noticed in our collection of breast cancer and melanoma samples, compared with normal external reference controls, was also characterized by a large interindividual variability. In order to confirm RINF overexpression in another malignant pathology, we determined RINF mRNA expression levels in thyroid cancer (Figure 2) for which paired samples of tumor and nontumor thyroid tissue were available from each patient (n = 54). Once again, the relative RINF mRNA expression was significantly higher in the thyroid tumor samples when compared with their nontumor counterparts, indicating that RINF is overexpressed in thyroid cancers (P < 0.001; Wilcoxon signed rank test for paired data).

RINF expression varies between tissues

When comparing the expression levels of RINF in the nontumor samples, significant differences were observed between normal tissues (P < 0.001; Kruskal–Wallis test) with thyroid tissue expressing, on average, approximately five-fold higher levels than breast and nevi. However, a significant difference was also observed between the latter two, with breast tissue expressing higher levels than nevi (P = 0.004; Mann–Whitney rank test).

No gene copy number change or mutations in RINF

Many genetic, epigenetic, and cellular changes are known to lead to alterations in gene expression levels. One of the most described events known to lead to overexpression of oncogenes like HER2 [15], MYCN [16], hDM2 [17], Cyclin D1 [18], and cdks [19] is an increase in the gene copy number. In order to confirm or exclude this mechanism as a cause for RINF overexpression in our cohort of solid tumors, we carried out...
The comparison of RINF expression in tumor versus matched benign patient (carried out at least in triplicates. Each dot corresponds to the ratio of one mRNA expression in the 108 samples and all the measurements have been normalized to the ribosomal RPLP2 gene. mRNA expression has been normalized to the ribosomal RPLP2 gene.

Since malignant melanoma and thyroid cancer are not available; data not shown). Among the 35 breast cancers analyzed, no reduced copy numbers were observed, whereas five samples displayed single duplications of the RINF locus. However, no association was observed between RINF mRNA expression level and relative gene copy number. This indicates that in most of our samples, the RINF mRNA overexpression most likely is the consequence of increased transcription of this gene rather than gene amplification. It cannot, however, be excluded that RINF gene amplification indeed exists in a few of our samples. Moreover, and in agreement with an increased RINF mRNA expression in these tumors, no mutation was found within any of the RINF open reading frames of 102 of the 124 tumor samples investigated in the present study (22 of the 57 breast cancer samples were not sequenced because their gDNA were not available; data not shown).

**RINF overexpression is associated with poor prognosis in locally advanced breast cancer**

Since a positive association was found between high RINF expression and the tumor state, we examined the potential value of RINF expression as a prognostic factor for overall survival. Since malignant melanoma and thyroid cancer are characterized by highly unfavorable and favorable prognosis, respectively, our datasets were too small to evaluate the prognostic impact of RINF expression in these malignancies. In contrast, compared with the two former diseases, breast cancer has an intermediate and more heterogeneous survival rate. Stratifying patients into those above versus below median expression value, we observed a nonsignificant trend toward poor survival among patients expressing high RINF levels ($P = 0.134$; log-rank test; Figure 4A). Interestingly, the poor survival was linked to the patients expressing particularly high levels of RINF. Stratifying breast cancer patients into three groups expressing low, intermediate, and high levels of RINF ($n = 19$ in each group), the low and intermediate group show no difference in survival, whereas a poor survival was recorded in the group expressing high RINF levels ($P = 0.045$; log-rank multiple comparison; $P = 0.013$, log-rank, high group versus the sum of low and intermediate groups; Figure 4B and C).

**RINF overexpression is associated with poor prognosis in confirmatory datasets**

In order to validate our data in additional cohorts of breast cancer patients, we searched for independent gene expression microarrays for which both survival and CXXC5 (RINF) mRNA expression data were available. Two large studies (accessible at Gene Expression Omnibus repository with the accession numbers GSE3494 and GSE6532) providing such data included patients from three independent institutions [12, 13]. The patients’ characteristics are summarized in supplemental Tables 4 and 5 (available at Annals of Oncology online). Notably, in these cohorts, the patients were not restricted to locally advanced cancers; in addition, the datasets contained tumors mutated as well as wild-type for TP53. For the GSE6532 dataset, overall survival data were not available; hence, DMFS was used as a surrogate marker.

Similar to what we observed in our own study, stratifying patients as above or below median RINF mRNA expression levels, we found a nonsignificant ($P = 0.098$, $P = 0.131$; log-rank; Figure 5A and D) or significant ($P = 0.004$; Figure 5G) trend towards poorer survival among the patients expressing high levels of RINF. Consistent with our own data, the three datasets revealed a poorer survival for the third of patients expressing the highest levels of RINF compared with the sum of the intermediate and low groups ($P = 0.043$, $P = 0.016$, $P = 0.001$; log-rank; Figure 5C, F, I). Considering breast cancer to be a heterogeneous disease, it was of interest to examine the levels of RINF in groups stratified by available molecular parameters known to be important for the tumor biology. Thus, we stratified the breast cancer patients according to estrogen receptor (ER) status. In spite of a lower number of patients, the observation of unfavorable prognosis among patients with high levels of RINF was still statistically convincing in the ER+ patients subgroup, both in our own patient cohort (supplemental Figure 2, available at Annals of Oncology online; $n = 45$; overall survival, $P = 0.003$) and the two independent studies (supplemental Figure 3, available at Annals of Oncology online), GSE3494 ($n = 199$; $P = 0.016$) and GSE6532 ($n = 33$; $P = 0.006$).

In order to evaluate RINF as a prognostic factor independent of treatment, we stratified the Loi dataset into Tamoxifen-treated ($n = 196$) and non-tamoxifen-treated ($n = 69$) patients (Figure 5). When dividing these patients into three groups according to RINF expression level, we observed a significantly poorer survival among the third of patients with the highest
RINF expression independent of tamoxifen therapy
(nontreated, $P = 0.016$; tamoxifen treated, $P = 0.001$; log-rank; Figure 5F). These results clearly indicate that the RINF expression level was a prognostic factor independent of the tamoxifen treatment.

RINF overexpression is associated with TP53 status in breast cancer

In order to explain why high RINF mRNA expression was associated with poor prognosis, we searched for putative statistical links between this index and other important patient’s clinical parameters known to be positively or negatively associated with prognosis in breast cancer (summarized in supplemental Table 1, available at Annals of Oncology online). In our cohort of patients, no significant correlations were found except for an association between high levels of RINF expression and estrogen receptor–positive tumors ($P = 0.020$; Mann–Whitney rank test). However, all 57 breast cancer patients included in our study are TP53 wild-type, so, in order to stratify the data according to this well-established tumor suppressor known to be associated with a worse overall and disease-free survival, independently of other risk factors, and that have also been implicated in resistance to anticancer therapies [20], we used one of the datasets that were used for verification of survival analyses [12]. Importantly, a strong correlation was found between RINF expression levels and TP53 mutation status, with high RINF being linked to TP53 wild type and low RINF linked to mutated TP53 ($P = 0.002$; Mann–Whitney rank test). These data indicate that RINF overexpression might be an alternative tumorigenic mechanism to TP53 mutations for the etiology of some breast cancers. This association between low RINF and TP53 mutation was even stronger when using the TP53 gene expression signature (diagonal linear discriminant analysis) as described by Miller et al. [12] ($P = 10^{-9}$; Mann–Whitney rank test). Further, the high RINF levels seem to be associated with a worse prognosis in both TP53 mutated and wild-type groups (supplemental Figure 4, available at Annals of Oncology online), suggesting this gene’s role as a prognostic factor to be independent of TP53 status.

Regarding another important prognostic factor in breast cancer, HER2-status, only 7 of the 57 patients analyzed here displayed staining index of 2 or 3 (data not shown). No effect of HER2 status was observed in a covariate analysis with RINF ($P = 0.923$).

discussion

The goal of this study was to investigate the expression pattern of RINF and its possible correlation to various clinical
parameters in solid tumors derived from tissues with diverse germ layer origins. As previous work on RINF has been carried out in leukemia systems (myeloid tissues from mesoderm), we chose to investigate solid tumors from breast and skin (derived from ectoderm) and thyroid (derived from endoderm).

For all three types of solid tumors, we found a significant overexpression of RINF in the malignant tissue compared with the nontumor tissue counterpart. The overexpression of RINF in malignant tissue was surprisingly contrasting to what was previously found in the NB4 leukemia cell line [1] where increased expression was linked to the nonmalignant phenotype (terminally differentiated cells). However, many genes have previously been observed to be differently regulated in leukemia and solid tumors, and interestingly, the tumoral versus normal deregulation of RINF expression shares common characteristics with deregulation of other important genes of differentiation, like ID1 [21, 22] and MAF [23], whose either up- or downregulation are functionally required according to the cell context and the differentiation program. Our present data could be part of an emerging concept that some particular genes of differentiation could functionally contribute to neoplasia by being up- or downregulated by epigenetic events rather than activating mutations, translocations, or gene amplification. Indeed, in contrast to most of the genes deregulated in cancer [24], genes of differentiation could be adversely regulated according to the tissue context.

Many microarray studies have been carried out over the last years in order to identify genes deregulated in cancer cells. However, most of the time, hundreds or thousands of genes are characterized at the same time and constitute altogether a gene signature associated with a molecularly defined disease. Even if these clusters of genes can be informative for prognosis, gene expression profiles have so far not been implemented as predictive factors regarding sensitivity to different chemotherapeutic regimens [25] except for the Oncotype DX analyzing expression of 21 genes with qPCR. The Oncotype DX-score reveals a moderate correlation to sensitivity for conventional treatment with cyclophosphamide, methotrexate, and 5-fluorouracil [26] as well as anthracycline-containing chemotherapy [27]. Here, we describe a single gene that was easily detectable by quantitative RT-PCR in all the malignant and benign samples investigated (about 220 samples in the

Figure 4. High retinoid-inducible nuclear factor (RINF) messenger RNA (mRNA) expression is related to bad prognosis in locally advanced breast tumors (n = 57). In our cohort of patients suffering from breast cancer (n = 57), the cumulative survivals (higher panels) and the relapse-free survivals (lower panels) are represented according to the RINF expression levels (Kaplan–Meier method). Patients have been classified in two equivalent groups (A), above (high, n = 28) or below (low, n = 28) the median RINF expression (as the number of patient was odd (57), the patient at the median has been censored, or in three equivalent groups (B) and (C), according to a high (n = 19), an intermediary (n = 19), or a low (n = 19) RINF expression. P values (log-rank test) of the comparison of the various groups of patients are indicated in the panels.
The present study) and that by itself is informative for prognostic of survival and recurrence in both early- and late-stage breast cancer disease and in different therapeutic settings.

RINF is a gene that until now has remained uninvestigated clinically, partly because its gene sequence was not known until recently, and consequently, its expression was not detectable by most of the conventional methods of genomics like microarray technologies. Indeed, its sequence has been first suggested as a putative gene, by in silico methods, because of its homology with other genes harboring a CXXC subtype zinc finger domain [28].

So far, little is known about the molecular mechanisms through which RINF executes its biological function. Very recently, a functional link has been suggested between RINF expression and two other important signaling pathways of differentiation that are known to often be deregulated in tumors, the beta-catenin/WNT [9, 29], and WT1 [30]. Another link has also been suggested at the molecular level between RINF and ATM-dependant transcriptional activation of TP53 [8]. These factors, with well-established roles in cancer, underline the importance to further investigate RINF functions in tumor tissues in order to better understand why RINF is a prognostic factor for breast cancer.

The finding here that high levels of RINF are associated with wild-type TP53 may indicate that RINF levels could substitute for TP53 mutations as a poor prognostic marker in breast cancer.

Even if further functional investigations will be required to prove the genuine involvement of this gene in cancer development, our results bring to light that RINF may play an important role in the process of tumorigenesis and high levels...
of RINF expression may be related to the malignant potential of tumor cells. Also, RINF may represent a potential biomarker for the diagnosis of these malignancies.

acknowledgements

We are grateful to all the patients who participated in the studies. F.P. thanks European commission and INSErM. We thank Genosplice technology (www.genosplice.com) for expert advice/help with data extraction from microarray datasets. Parts of this work have been carried out at Mohan Cancer Research Laboratory.

funding

This work was supported by grants from the Norwegian Research Council and the University of Bergen. S.K. is a recipient of a postdoctoral fellowship from the Norwegian Cancer Society, L.M.M. and C.B. are recipients of PhD fellowship from the Norwegian Cancer Society.

disclosure

Some of the technologies used in this work are the basis for a patent application filed by S.K., T.A., J.R.L., and F.P. through a patent application filed by S.K., T.A., J.R.L., and F.P. through the University of Bergen. Other than this, no competing interests are declared.

references