Histol Histopathol (2020) 35: 1181-1187

http://www.hh.um.es

Histology and Histopathology
From Cell Biology to Tissue Engineering

Combined expression levels of KDM2A and KDM2B correlate with nucleolar size and prognosis in primary breast carcinomas

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Summary. Ribosome biogenesis is a fine-tuned cellular process and its deregulation is linked to cancer progression: tumors characterized by an intense ribosome biogenesis often display a more aggressive behavior. Ribosomal RNA (rRNA) synthesis is controlled at several levels, the higher one being the epigenetic regulation of the condensation of chromatin portions containing rRNA genes. KDM2A and KDM2B (Lysine (K)-specific demethylase 2A/B) are histone demethylases modulating the accessibility of ribosomal genes, thereby regulating their transcription. Both enzymes are able to demethylate lysins at relevant sites (e.g. K4, K36) on histone H3. We previously demonstrated that KDM2B is one of the factors regulating ribosome biogenesis in human breast cancer. In this study we aimed to define the combined contribution of KDM2A and KDM2B to breast cancer outcome. KDM2A and KDM2B mRNA levels, nucleolar area as a marker of ribosome biogenesis, and patients' prognosis were retrospectively assessed in a series of primary breast carcinomas. We observed that tumors characterized by reduced levels of both KDM2A and KDM2B displayed a particularly aggressive clinical behavior and increased nucleolar size. Our results suggest that KDM2A and KDM2B may cooperate in regulating ribosome biogenesis thus influencing the biological behavior and clinical outcome of human breast cancers.

Key words: Breast Cancer, Histone modification, Ribosome biogenesis, Prognosis

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DOI: 10.14670/HH-18-248

Introduction

The biosynthesis of ribosomes (ribosome biogenesis) is a complex and highly coordinated cellular process, which starts in the cell nucleolus and ends in the cytoplasm (Kressler et al., 2010; Thomson et al., 2013). An adequate rate of ribosome biogenesis ensures the conservation of the proper ribosomal allocation and protein synthetic potential in the cell. As a consequence, ribosome biogenesis is constantly up-regulated in proliferating cells and its deregulation is linked to neoplastic progression (Montanaro et al., 2008). Indeed, cancer cells almost constantly display an increased rate of ribosome production which, from the morpho-functional standpoint, results in an increased nucleolar size (Derenzini et al., 2009). In this regard, the size of the nucleolus after its selective silver staining has been recognized by several studies as a very strong prognostic indicator in a variety of human tumor types (Derenzini et al., 2009).

A limiting step in ribosome biogenesis is represented by the synthesis of ribosomal RNA (rRNA). rRNA synthesis is controlled at several levels, the higher one being the epigenetic regulation of the condensation of chromatin portions containing rRNA genes (Grummt and Längst, 2013). Human cells contain hundreds (about

400) of rDNA gene copies organized as tandem, head-to-tail repeats (Kopp et al., 2007; Lempiäinen and Shore, 2009). During metaphase rDNA is located on the short arms of acrocentric chromosomes; during interphase these chromosomal portions, called nucleolar organizer regions (NORs), fuse to form the nucleolus (Sirri et al., 2000; Hernandez-Verdun, 2006).

The transcription of rDNA is operated by RNA polymerase I (Pol I) and this process is finely regulated at the epigenetic and transcriptional levels. Concerning the epigenetic modifications regulating transcription, aside from the better-known process of CpGs methylation, there are other chemical modifications on different aminoacidic residues of histone tails: acetylation, methylation, phosphorylation and ubiquitylation. These modifications can regulate the switch from active rDNA repeat clusters to non-active ones: an open chromatin structure is generally characterized by histone H3 and H4 acetylation (H3ac and H4ac), histone H3 di- or trimethylation at lysine 4 (H3K4me2, H3K4me3) and mono-, di- and trimethylation at lysine 36 (H3K36me, H3K36me2, H3K36me3) (Lawrence et al., 2004; Grummt and Längst, 2013).

KDM2A and KDM2B (Lysine (K)-specific demethylase 2 A/B) are histone demethylases, harboring the JmjC domain, modulating the accessibility of rDNA genes, thereby repressing their transcription. Both enzymes are able to demethylate lysins at relevant and specific sites on histone H3: KDM2B is involved in the demethylation of lysine 4 trimethylated site (H3K4me3) and lysine 36 dimethylated site (H3K36me2), whereas KDM2A targets are monomethylated lysine residues in position 36 on histone H3 (H3K36me1) or demethylated ones in position 36 on histone H2 (H2K36me2) (Tsukada et al., 2006; Pan et al., 2012; Rizwani et al., 2014; Tanaka et al., 2015). The expression of both these enzymes has been demonstrated to influence rRNA transcription and ribosome biogenesis in human cells (Frescas et al., 2007; Tanaka et al., 2015).

We previously demonstrated that KDM2B is one of the factors regulating ribosome biogenesis in human breast cancer and that its reduced expression is associated with enhanced rRNA transcription and increased nucleolar size in breast cancer cells as well as significantly reduced patients' disease-free survival (Penzo et al., 2015; Galbiati et al., 2017). In this study, on the basis its functional similarities with KDM2B, we aimed to define the contribution of KDM2A on ribosome biogenesis and tumor prognosis in a series of human primary breast carcinomas.

Materials and methods

Patients' material

A total of one hundred and seventy breast carcinomas were selected from a series of consecutive patients who had undergone surgical resection for primary breast carcinoma at the Surgical Department of the University of Bologna on the sole basis of frozen tissue availability for KDM2A and KDM2B mRNA expression determination. Part of the cases were obtained from a previous study (Montanaro et al., 2006) while additional samples were collected after 2011.

Data on tumor histological classification, grading, size and TNM classification were obtained as described (Montanaro et al, 2006). Surrogate bioprofile classification of the cases on the basis of histological results was performed according to St. Gallen 2017 consensus (Curigliano et al., 2017). Informed consent was obtained from all individual participants included in the study.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Research Committee (n. 75/2011/U/Tess approved on 7/19/2011 and 132/2015/U/Tess approved on 10/13/2015 by Policlinico S.Orsola - Malpighi Ethical Review Board, Bologna, Italy) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Real-time RT-PCR

Total RNA was extracted from frozen samples using Genelute RNA/DNA/protein Plus Purification Kit (Sigma-Aldrich, USA). 1 μg of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, USA), following the manufacturer's instructions. The cDNA was subjected to real-time PCR using a CFX Connect thermocycler (Bio-Rad) with TaqMan probes in these cycling conditions: 95°C for 3 min, 40 cycles at 95°C for 10 sec and 55°C for 30 sec.

The sets of primers and fluorogenic probes used for KDM2A were purchased from Thermo Scientific (KDM2A probe: Hs00957938). Quantitative (q) RT-PCR reactions were run in triplicate for each analyzed target. The expression of the KDM2A gene was calculated using human β-glucuronidase (Thermo Scientific) as housekeeping target and the cDNA of MCF7 cell line as calibrator. Threshold cycles (Ct) in each triplicate were averaged and fold differences were calculated by the $\Delta\Delta$Ct method (Dussault and Pouliot, 2006). KDM2B expression values were previously obtained on the same specimens (Penzo et al., 2015).

Selective silver-staining of nucleoli

Tissue slices were deparaffinized and re-hydrated; antigen retrieval was performed in autoclave in citrate buffer at pH 6. Silver staining was performed following to the "one step" method originally described by Ploton et al. (1986). Silver staining was carried out for 13 min at 37°C using a solution of silver nitrate at 30% (w/v) in 2% gelatin, 1% aqueous formic acid. After extensive washing in water, tissue slices were dehydrated and mounted with coverslips. For each slide, 5 images were captured and nucleolar size was measured using Image

Pro Analyzer software.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software package (Statistical Package for Social Science, SPSS Inc, Chicago, IL). Correlations between continuous variables were computed by means of the Spearman rank correlation test. Differences among groups were evaluated using the Mann- Whitney U-, or Kruskal-Wallis test, as appropriate. Univariate analysis for disease-free survival (DFS) was performed using the Kaplan and Meier approach and the differences between curves were tested using the log-rank test. p values below 0.05 were regarded as significant.

Results

KDM2A mRNA levels showed a high variability in the studied tumor samples (170 cases, range 0-19.97, mean 1.45, SD 2.37, median 0.91 arbitrary units - a.u.).

Table 1. KDM2A mRNA levels and clinical and biopathological parameters.

	KDM2A mean ± SD	n	p
Histology			0.790
Ductal	1.36±2.14	133	
Lobular	2.23±3.83	22	
Others	1.47±1.24	5	
N			0.638
N0	1.38±2.22	67	
N+	1.40±1.79	80	
T			0.011
T1	1.29±2.49	81	
T2-4	1.74±2.38	76	
Nuclear Grade			0.32
GN1	2.49±4.27	18	
GN2	1.43±2.94	52	
GN3	1.29±1.34	89	
Estrogen Receptor			0.155
Negative	1.33±0.92	34	
positive	1.95±2.37	84	
Progesteron Receptor			0.420
Negative	1.53±1.36	65	
positive	2.06±2.69	53	
Ki67 LI			0.726
<20 %	1.25±1.62	69	
≥20 %	1.43±2.16	88	
p53			0.756
negative	1.35±1.98	126	
positive	1.36±1.78	31	
Her2			0.102
negative	1.40±2.37	94	
positive	1.16±0.87	56	

SD, standard deviation; n, number; p, p value; N, lymph node involvement: N0, no lymph node involvement; N+, lymph node involvement; T, size of tumor: T1≤2 cm; T2-4>2 cm; NG, nuclear grade; LI, Labelling Index.

Correlations among KDM2A mRNA levels and common clinical and bio-pathological parameters are reported in Table 1. Among the different variables considered, KDM2A mRNA levels resulted to be significantly associated with the tumor size at diagnosis (p=0.011).

In this tumor series, KDM2A mRNA levels turned out to be significantly related also to the KDM2A cognate gene KDM2B (r=0.373, p<0.001). In a previous study on the same series we observed a significant inverse linear correlation between the expression of KDM2B mRNA and nucleolar size measured in cancer cells after selective silver staining (Penzo et al., 2015). Data on nucleolar area were available for 122 cases (range 1.53-9.84, mean 4.32, SD 1.88 μm^2). Similarly to what was reported for KDM2B, the inverse linear correlation was confirmed also between nucleolar size and KDM2A mRNA levels (r=-0.193, p=0.033 - See Fig. 1).

We then evaluated the relationship between KDM2A expression and prognosis in our series. Follow-up data were available for 95 patients (mean follow-up= 62.36±37.38 SD, range 1-129 months). On the basis of KDM2A mRNA levels, we arbitrarily divided the cases into two groups: one with low KDM2A values (n=57) and a second group with high KDM2A values (n=38), choosing the cut-off (1.60 a.u.) which allowed us to obtain the highest predictive value in terms of disease-free survival (DFS). Survival analysis showed that tumors with high KDM2A expression were characterized by a higher (87.2%) DFS than tumors of the other group (66.7%-χ^2=6.75, p=0.01 - Fig. 2a). Therefore, higher KDM2A levels appeared to be associated with a more favorable outcome. These results are in keeping with those obtained for KDM2B in a previous study on the

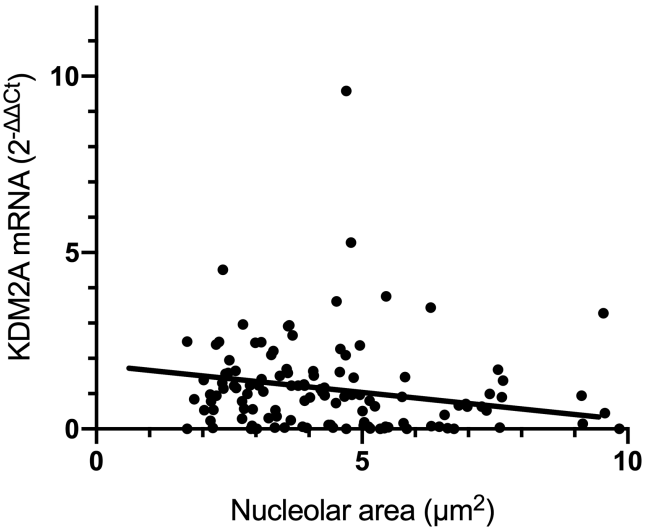

Fig. 1. KDM2A mRNA levels are related to nucleolar area in breast cancer. KDM2A expression by qRT-PCR and nucleolar area after selective staining with silver and morphometric analysis in 122 primary breast carcinomas. A significant inverse linear correlation was observed (r=-0.193, p=0.033).

same series (Penzo et al., 2015). In addition, we evaluated the prognostic value of KDM2A mRNA levels in the cases which were grouped based on their bio-profile classification. Interestingly, in tumors classified as luminal B (n=24) low KDM2A levels turned out to be significantly associated with shorter DFS (58.3%) as compared to those expressing higher KDM2A levels (91.7%-χ^2=4.23, p=0.04 - Fig. 2b). In the other bio-profile groups considered (triple positive, Luminal A, triple negative), although cases with high KDM2A expression were constantly characterized by a better prognosis, no significant prognostic value was found.

Since KDM2A and KDM2B are endowed with very similar activities, in particular they both act by demethylating H3K36me2, we investigated the clinical behavior of tumors expressing low levels of both enzymes. We then combined our KDM2A data with the data collected in our previous study for KDM2B expression (Penzo et al., 2015) conducted on the same series, and we compared the survival of those cases expressing low KDM2A and low KDM2B mRNAs with that of all remaining cases (high KDM2A/high KDM2B, high KDM2A/low KDM2B, low KDM2A/high KDM2B). The tumors characterized by low levels of both demethylases (n=40) displayed a particularly unfavorable prognosis (DFS=59.1%) in comparison to all remaining cases (n=51 - DFS 83.9% χ^2 10.11, p=0.001 - Fig. 3). The prognostic value of this combined evaluation was strikingly related to prognosis in the luminal B bio-profile group (low KDM2A and low KDM2B, n=6, DFS 33%, others, n=17, 88.2%-χ^2 10.19, p=0.001 - Fig. 3). No significant association between the KDM2A/KDM2B combined evaluation levels and DFS was found in the other bio-profile groups.

We also evaluated the relationship between nucleolar area after selective silver staining and prognosis in our

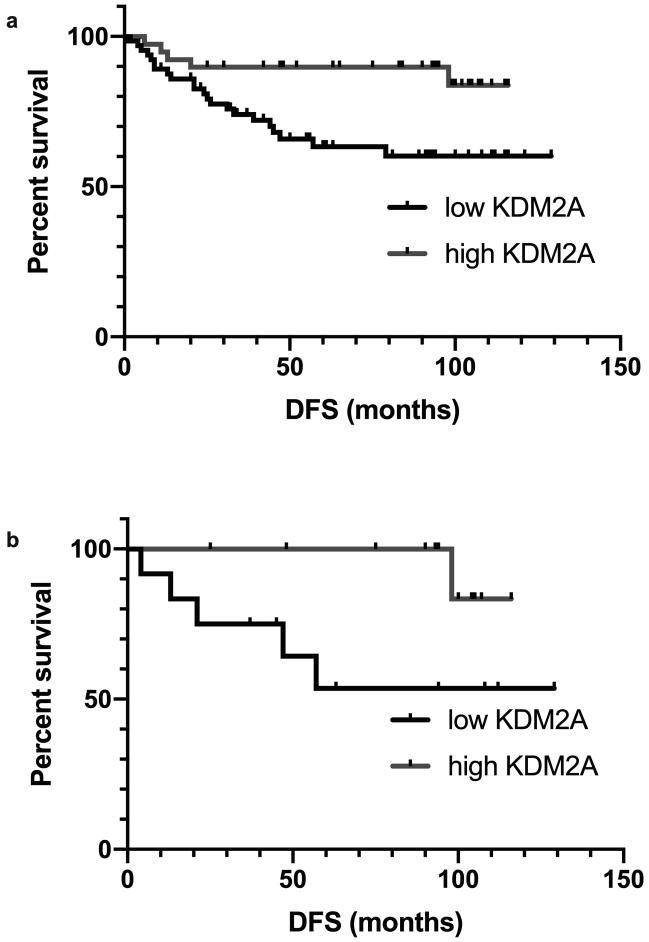

Fig. 2. KDM2A mRNA levels are related to survival in breast cancer. **a.** Primary breast cancer with high KDM2A mRNA expression showed a better (87,2%) disease free survival (DFS) than tumors with lower KDM2A mRNA expression (66.7%), (χ^2=6.75, p=0.01). **b.** DFS in luminal B breast carcinoma divided according to KDM2A mRNA levels (91.7% in high KDM2A mRNA group vs. 58.3% in KDM2A mRNA group low, χ^2=4.23, p=0.04).

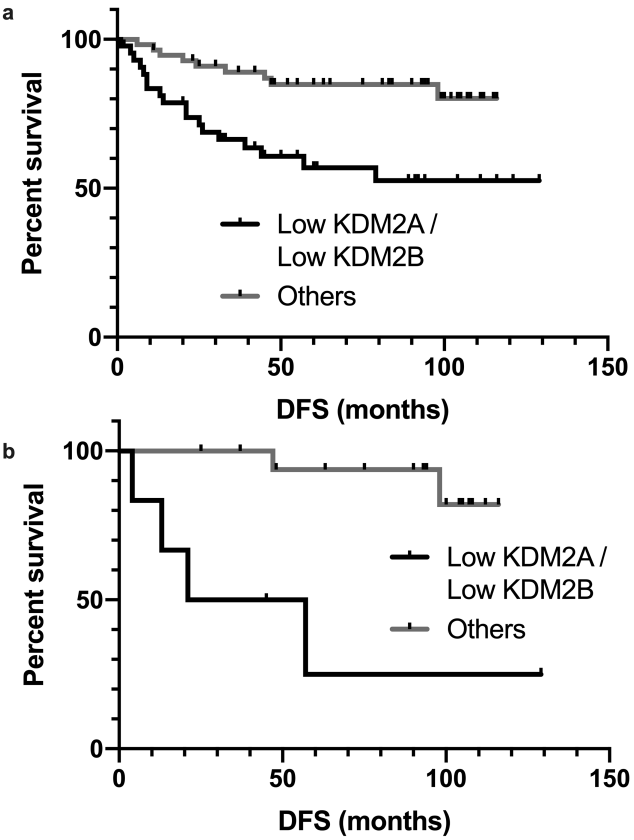

Fig. 3. KDM2A/KDM2B combined evaluation is related to survival in breast cancer. **a.** Primary breast cancers with low KDM2A and low KDM2B mRNA expression showed a worse (59.1%) disease free survival (DFS) than other cases (83,9%), (χ^2=10.11, p=0.001). **b.** DFS in luminal B breast carcinoma divided according to combined KDM2A/KDM2B mRNA levels (33.3% in low KDM2A/low KDM2B mRNA group, 88.2% in all remaining cases, χ^2=10.19, p=0.001).

series. In this case, follow-up data were available for 72 patients (mean follow-up= 48.94±35.03 SD, range 1-115 months). We divided the cases into two groups: one with low nucleolar area values (<3 μm^2, n=24) and a second group with large nucleolar area (≥3 μm^2, n=48). Survival analysis showed that tumors with low nucleolar area were characterized by a higher (88,0%) DFS than tumors of the other group (67.3%), (log-rank test χ^2 3.77, p=0.05 - Fig. 4A).

Finally, we compared the nucleolar size of the two groups resulting from the KDM2A/KDM2B combined evaluation. The mean size of the nucleolus of tumors characterized by low KDM2A/KDM2B values (4.88±2.08 SD μm^2) resulted significantly larger than in the remaining cases (3.85±1.53 SD μm^2- p=0.008 - Fig. 4B).

Discussion

In the present study we found that KDM2A mRNA levels are inversely associated with the nucleolar area in human breast carcinoma specimens. The expression of the KDM2A mRNA was also associated with patients' prognosis. Interestingly, when analyzing the prognostic impact of KDM2A expression in relationship with the expression of its cognate gene KDM2B, we observed that tumors characterized by a reduced expression of both these genes are characterized by a particularly unfavorable prognosis.

The association between KDM2A and nucleolar size is well in line with its known role in the control of rRNA transcription. Indeed, KDM2A is known to be an epigenetic negative regulator of rDNA transcription, for instance after nutrient starvation (Tanaka et al., 2010; Tanaka et al., 2015). Since in tumors the nucleolar size is a morphological indicator of the rate of ribosome biogenesis (Derenzini et al., 2009) and that one recognized limiting step for ribosome biogenesis is represented by rDNA transcription (Lempiäinen and Shore, 2009), it is not surprising that these two variables have some relationship in human tumors.

Such a relationship also provides an explanation for the inverse association observed between KDM2A mRNA levels and patients' specific prognosis. Increased nucleolar size is indeed a very well- recognized negative prognostic factor in breast cancer (Ceccarelli et al., 2000) (similarly to what occurs in many other cancer types) (Derenzini et al., 2009). Conversely, the prognostic value of KDM2A cannot be ascribed to is relationship with the tumor size at diagnosis, since bigger tumors were typically characterized by higher KDM2A mRNA levels.

These results are however in contrast with those of a study linking KDM2A levels and prognosis in triple-negative breast cancer (Chen et al., 2017). Inconsistency may be in part explained considering the difference in the series (in our series triple negative are only 12,4% of the total) and in the method for evaluating of KDM2A (qRT-PCR vs immunohistochemistry).

Our results are also in contrast with those reported by Xu et al. (2019), who showed an inhibition of proliferation upon KDM2A knockdown in kidney cells. Again, the contrasting results may be explained with the differences in cancer type (kidney vs breast cancer) and

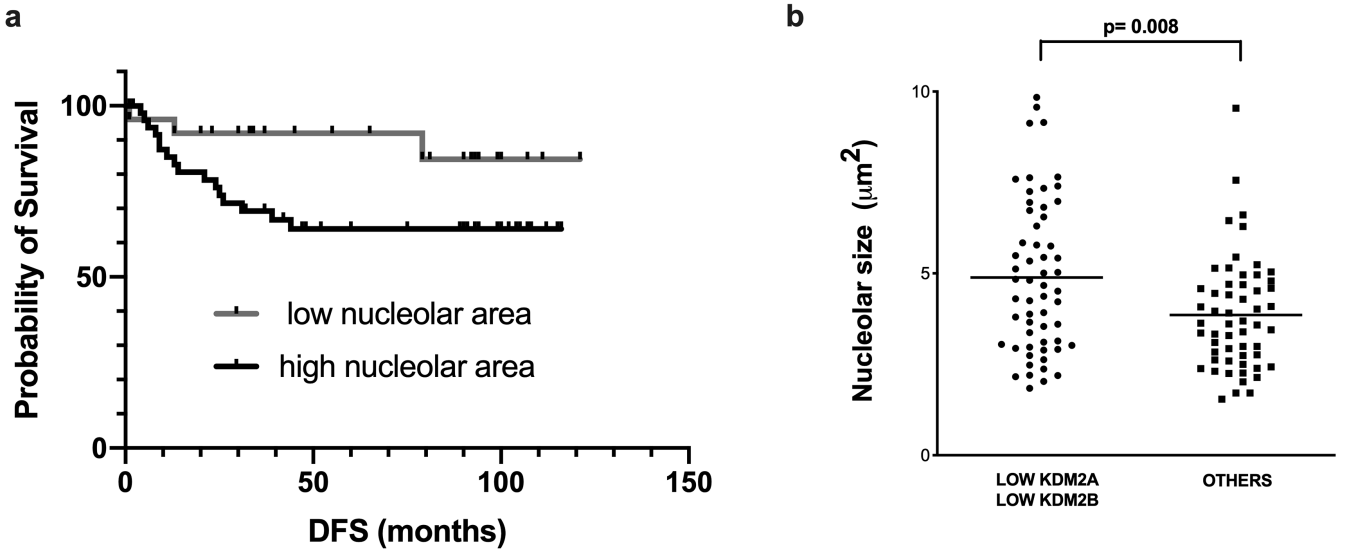

Fig. 4. Nucleolar Area, prognosis and combined KDM2A/KDM2B evaluation. **a.** Nucleolar area is significantly related to prognosis in the studied breast cancer series: high nucleolar area tumors (≥3 μm^2, n=48) are characterized by worse disease-free survival (DFS) than those with low nucleolar area c (<3 μm^2, n=24 - log-rank test χ^2 3.77, p=0.05). **b.** The size of the nucleolus of tumor cells characterized by low KDM2A/KDM2B values (4.88±2.08 SD μm^2) is significantly higher than those of remaining cases (3.85±1.53 SD μm^2-p=0.008).

cellular models (*in vitro* HEK293T cells vs *ex vivo* patients breast cancer cells). Moreover, in this specific study a different method was used for the evaluation of KDM2A expression (immunohisto-chemistry vs qRT-PCR).

The results presented herein are well in line with what we have previously described for KDM2B in the same series (Penzo et al., 2015), and they are consistent with the similar activities of the two histone demethylases which also share common targets (Martin and Zhang, 2005; Grummt and Längst, 2013). On these bases the strong predictive prognostic significance of the combined evaluation of KDM2A and KDM2B may find justification; indeed, since they both act on a common target, when both histone demethylases are lacking this may result in a lack of removal of the methyl group on those residues which are pivotal for epigenetic control.

It is worth considering the prognostic impact of both KDM2A mRNA levels and the KDM2A/KDM2B combined evaluation in breast cancer cases classified as luminal B. This particular subtype is indeed characterized by an intermediate prognosis and different therapeutic options. One particular feature of luminal B breast cancer, as compared, for instance, to luminal A cases, is the higher Ki67 labelling index, i.e. an increased fraction of proliferating cancer cells. On the other hand, in proliferating cells the rate of ribosome biogenesis and nucleolar size are strongly related to the rapidity of cell proliferation (Montanaro et al., 2008). It is not surprising therefore that the strongest prognostic value of a factor influencing ribosome biogenesis is observed in a class of tumor characterized by a high percentage of proliferating cells. The results of this study may therefore pave the way to the use of histone modifier (such as KDM2A and KDM2B) expression as a useful marker in the risk assessment of patients with luminal B breast carcinomas.

The concomitant presence of low expression levels of KDM2A and KDM2B mRNAs and increased nucleolar size may then further suggest that the mechanism contributing to a more aggressive behavior of these tumors may derive from their upregulated ribosome biogenesis. In the case that this link is experimentally proven, it may be of interest to consider that a number of drugs commonly used for cancer treatment, and having a direct effect on rRNA transcription, may be particularly effective in tumors addicted to elevated rates of ribosome production (Montanaro et al., 2013; Penzo et al., 2019). Similarly, given the increased availability of a new generation of compounds specifically targeting the activity of histone modifiers (Yan et al., 2016), further studies may evaluate the effect of drugs specifically targeting the modifications resulting from the lack of KDM2A and KDM2B (e.g. by inhibiting the activity of the antagonist methyltransferases).

In conclusion, our results suggest that KDM2A and KDM2B may cooperate influencing the biological behavior and clinical outcome of human breast cancers possibly by regulating ribosome biogenesis. Based on our results the quantitative evaluation of the expression of the mRNAs for these two histone demethylases could be useful to obtain prognostic information, particularly in luminal B breast carcinomas.

Acknowledgements. LM and MP are grateful to Fondazione AIRC for its support (grants numbers IG15212 and MFAG19941, respectively). This study was supported by funds from Roberto and Cornelia Pallotti's legacy for cancer research to DT, LM, and MP.

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Accepted September 9, 2020