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Ribosomal RNA 2′O-methylation as a novel layer of inter-tumour heterogeneity in breast cancer

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ABSTRACT

Recent epitranscriptomics studies unravelled that ribosomal RNA (rRNA) 2′O-methylation is an additional layer of gene expression regulation highlighting the ribosome as a novel actor of translation control. However, this major finding lies on evidences coming mainly, if not exclusively, from cellular models. Using the innovative next-generation RiboMeth-seq technology, we established the first rRNA 2′O-methylation landscape in 195 primary human breast tumours. We uncovered the existence of compulsory/stable sites, which show limited inter-patient variability in their 2′O-methylation level, which map on functionally important sites of the human ribosome structure and which are surrounded by variable sites found from the second nucleotide layers. Our data demonstrate that some positions within the rRNA molecules can tolerate absence of 2′O-methylation in tumoral and healthy tissues. We also reveal that rRNA 2′O-methylation exhibits intra- and inter-patient variability in breast tumours. Its level is indeed differentially associated with breast cancer subtype and tumour grade. Altogether, our rRNA 2′O-methylation profiling of a large-scale human sample collection provides the first compelling evidence that ribosome variability occurs in humans and suggests that rRNA 2′O-methylation might represent a relevant element of tumour biology useful in clinic. This novel variability at molecular level offers

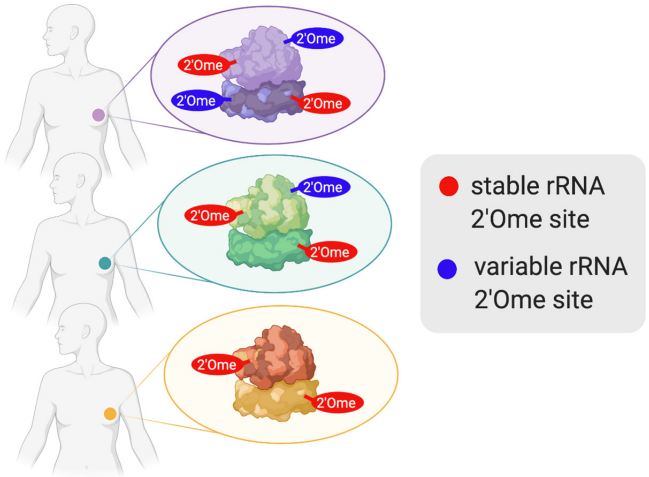
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an additional layer to capture the cancer heterogeneity and associates with specific features of tumour biology thus offering a novel targetable molecular signature in cancer.

GRAPHICAL ABSTRACT



INTRODUCTION

Emerging evidence suggests that changes in ribosomal RNA 2′O-ribose methylation (rRNA 2′OMe) in the human ribosome play a key role in regulating translation thereby contributing in setting particular phenotypes such as hallmarks of cancer cells (1–5). Using cancer cellular models, we and others indeed reported an association between variation in rRNA 2′O-methylation and modulation of translation efficiency of particular mRNA subsets in cancer (1,6–9). In particular, we demonstrated that inactivation of the tumour suppressor p53 protein in mammary epithelial cells is associated with an increased expression of the 2′O-methyltransferase *FBL*, an alteration of the 2′O-methylation level at some of the 106 rRNA-methylated sites and an increased translation of some cellular Internal Ribosome Entry Sites (IRES)-containing mRNAs such as those encoding the oncogenic IGF1R and cMyc proteins (1). Moreover, we have shown that *FBL* overexpression promotes IGF1R-dependent proliferation, colony formation and resistance to chemotherapy in breast cancer cell lines, suggesting a role of rRNA 2′O-methylation in mammary tumorigenesis and breast cancer progression (1). Importantly, using cell-free *in vitro* translation assays, we recently demonstrated that modulation of rRNA 2′O-methylation directly affects translation initiation of IRES-containing mRNAs, the IGF1R mRNA and the Cricket paralysis virus mRNA (2). This new layer of gene expression regulation recently uncovered, joins the numerous descriptions of chemical modifications of both coding and non-coding RNAs that regulates post-transcriptional processes, a field known as epitranscriptomics (3,4,10). However at present, it is unclear whether rRNA 2′O-methylation varies in humans.

Although genetics, genomics, epigenetics, transcriptomics and proteomics revealed the heterogeneity of breast tumours at the molecular level, identification of novel molecular layers are required to fully capture all the inter-patient variability, essential for developing clinical applications (11). Amongst them, epigenetics appears as promising additional molecular fingerprints to improve patient classification, prognosis or treatment, including in breast cancer (11–13). In these molecular portraits of breast cancers, RNA epitranscriptomics and more specifically rRNA 2′O-methylation, has never been investigated. Thanks to the recent resolution of technological issues, including the development of RNA-seq-based approaches dedicated to the profiling of rRNA 2′O-methylation (4,5,14,15) and the recent developments of high-resolution cryo-EM that has provided insights into the detailed structure of the human ribosome including rRNA modifications (16–18), rRNA 2′O-methylation profiling can now be achieved in human samples to determine whether it can provide a molecular fingerprint in breast cancer.

Here, we present the first rRNA 2′O-methylation landscape of human breast tumours. We used the next-generation RiboMeth-seq technology that we remodelled to perform high-throughput analyses of human clinical samples. Our data reveal the unexpected existence of specific modulation of 2′O-methylation at only particular rRNA positions between human breast tumours that are associated with clinical outcome and biological features.

MATERIALS AND METHODS

Human samples

A series of 195 female primary breast tumours were collected from 1997 to 2011 and maintained by the Tayside Tissue Bank (TTB, Dundee, Scotland, UK) under ethical approval (REC Reference 07/S1402/90) (Supplementary Table ST1) (19). This retrospective series of primary breast tumours is composed of both non-invasive (45%) and invasive (55%) lymph node tumours (Supplementary Table ST1). Total RNA was extracted from frozen tissues by TTB services as already described (19,20). A second series of 7 mammoplasties (i.e. non-transformed, non-proliferative but mostly adipocyte cells) derived from healthy donors was sequenced independently (Institut Curie, France) (21). A third series composed of 10 mammary malignant tumours and 8 mammary benign tumours (i.e. non-transformed but hyper-proliferative epithelial cells) were collected between 2006 and 2007 (Institut Gustave Roussy, France) from consenting patients using fine-needle aspiration after suspicion of breast lesion (Supplementary Table ST2) (22). A human RNA reference sample (i.e. RNA reference) was used as a calibrated source of rRNA (Human XpressRef Universal Total RNA, Qiagen) prepared from 20 different human adult and foetal normal major organs.

RiboMeth-seq

Levels of rRNA 2′O-methylation at the 106 rRNA 2′O-methylated sites were determined by RiboMeth-seq (14,15,23–25). Presence of 2′O-methylation protects the phosphodiester bond located at the 3′ of the 2′O-methylated nucleotide from alkaline hydrolysis. Thus, the presence of

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2′O-methylation at the given nucleotide *n* induces underrepresentation of RNA fragments starting at the nucleotide *n + 1* and ending at position *n* allowing to calculate a 2′O-methylation level at the corresponding nucleotide position (or C-score) varying from 0 to 1 (15). RiboMeth-seq was performed using the Illumina sequencing technology and raw data were processed as previously described (14,25). The median number of total reads reaches 7.2 millions after trimming, these reads being aligned on the 7.2 kb-long rRNA sequences, that corresponds to the optimal sequencing depth (14,26).

Analysis of the breast cancer series

Amongst an initial series of 214 primary breast tumours, RiboMeth-seq data of 195 primary breast tumour samples passed the QC criteria (representing 91% of the initial series) and were thus retained for the downstream analyses (Supplementary Table ST1). Unsupervised data analysis was performed (hierarchical clustering and principal component analysis (PCA)) using the C-scores at the 106 individual rRNA 2′O-methylated sites of the 195 samples. rRNA 2′O-methylation profiles were shown as either box-and-whiskers plots, line charts or barcharts. Classification of the rRNA 2′O-methylation sites into 'stable' and 'variable' classes was done empirically based on the variability of the sites (in terms of interquartile range, IQR), the cut-off site being the site from which the IQR values no longer lie on this straight line (see Supplementary Materials and Methods for details). Between-group comparisons were performed using Fisher's exact test for categorical data or Mann–Whitney test for quantitative data. Bonferroni or false discovery rate correction methods were applied for multiple comparisons (P.adj). All *P*-values corresponded to two-tailed *P*-values. A P-value < 0.05 was considered to be statistically significant. Statistical analyses and graphical representations were performed using either R v3.6.3 or GraphPad Prism v7.0a software (GraphPad Software, Inc).

rRNA 2′O-methylation evolution and mapping

To further characterize the two classes of human rRNA 2′O-methylated sites, their conservation during evolution was compared using box C/D snoRNA guides as surrogates of rRNA 2′O-methylated sites, since to date availability of rRNA 2′O-methylation profiles is limited to only few organisms. Three evolution groups were established using the snoRNA-LBME-db database (www-snorna.biotoul.fr). Using Fisher's exact test, enrichment analysis was performed to compare the proportion of stable versus variable sites amongst the three groups of rRNA 2′O-methylated sites. The rRNA 2′O-methylated sites were mapped on the structure of the HeLa cancer cell human ribosome determined by cryo-EM (16–18) and images were drawn using the PyMol software. Observations were based on previous reported three-dimensional molecular docking analysis of tRNAs or ribosome-associated factors, as already discussed in (ref 16–18).

RESULTS

Optimization of RiboMeth-seq technology for human samples

To profile rRNA 2′O-methylation in human primary breast tumours, we used the novel RiboMeth-seq technology. The measurement of 2′O-methylation frequency by RiboMeth-seq technology relies on a partial alkaline hydrolysis of the rRNA phosphodiester bonds, which become refractory to hydrolysis when adjacent riboses are methylated in position 2′ (14,15,25). RiboMeth-seq processing yields a score (i.e. C-score) at each of the 106 rRNA 2′O-methylated positions, reflecting the level of 2′O-methylation.

Since rRNA 2′O-methylation profiling has never been performed on large series of human samples, we developed RiboMeth-seq-dedicated extensive quality controls based on numerous metrics (see Supplementary Materials and Methods). Using human RNA reference samples composed of a mix of 20 different human adult and foetal normal major organs and a test set of 20 primary breast tumour samples, we show a strong correlation in the C-scores amongst the technical replicates (Supplementary Figures S1 and 2). Overall, our data demonstrate that the high-throughput RiboMeth-seq technology is a reproducible and robust technology even when sequencing delicate biological material such as frozen tumour biopsies.

Identification of two classes of rRNA 2′O-methylation sites

We then profiled rRNA 2′O-methylation in a series of 195 primary breast cancers composed of a mix of invasive and non-invasive tumours using RiboMeth-seq (Supplementary Table S1) (20). Unsupervised statistical analysis revealed variability in rRNA 2′O-methylation levels (Figure 1). It first showed that, for a given tumour, the 2′O-methylation level varies between 0 and 0.98 amongst the 106 rRNA 2′O-methylation sites (Figure 1 and Supplementary Figure S3a). This demonstrates that in a single human tumour sample, all rRNA molecules are not fully and equally 2′O-methylated at the 106 sites.

Hierarchical clustering also revealed that, for a given rRNA site, 2′O-methylation levels differ when comparing the 195 human tumours (i.e. inter-patient variability) (Figure 1; Supplementary Figures S3a and 4). We defined two classes of rRNA 2′O-methylated sites based on the variability of their 2′O-methylation level amongst patients (Supplementary Figure S3c). The classification into these two classes was done empirically based on the intra-variability of the sites (in terms of interquartile range, IQR, at a given site). The first class contains 60 rRNA 2′O-methylated sites (56.6% of all the rRNA 2′O-methylated positions), the levels of which exhibit low inter-patient variability between the 195 tumour samples, despite these series representing the full spectrum of breast cancer subtypes (Figure 2a and Supplementary Figure S4, red). Thereafter, they are termed 'stable' sites since they display the most stable C-scores between the 195 tumours. The second class corresponds to a limited number of rRNA 2′O-methylated sites (46 sites, around 43.4% of the rRNA 2′O-methylated positions) that exhibit high inter-patient vari-

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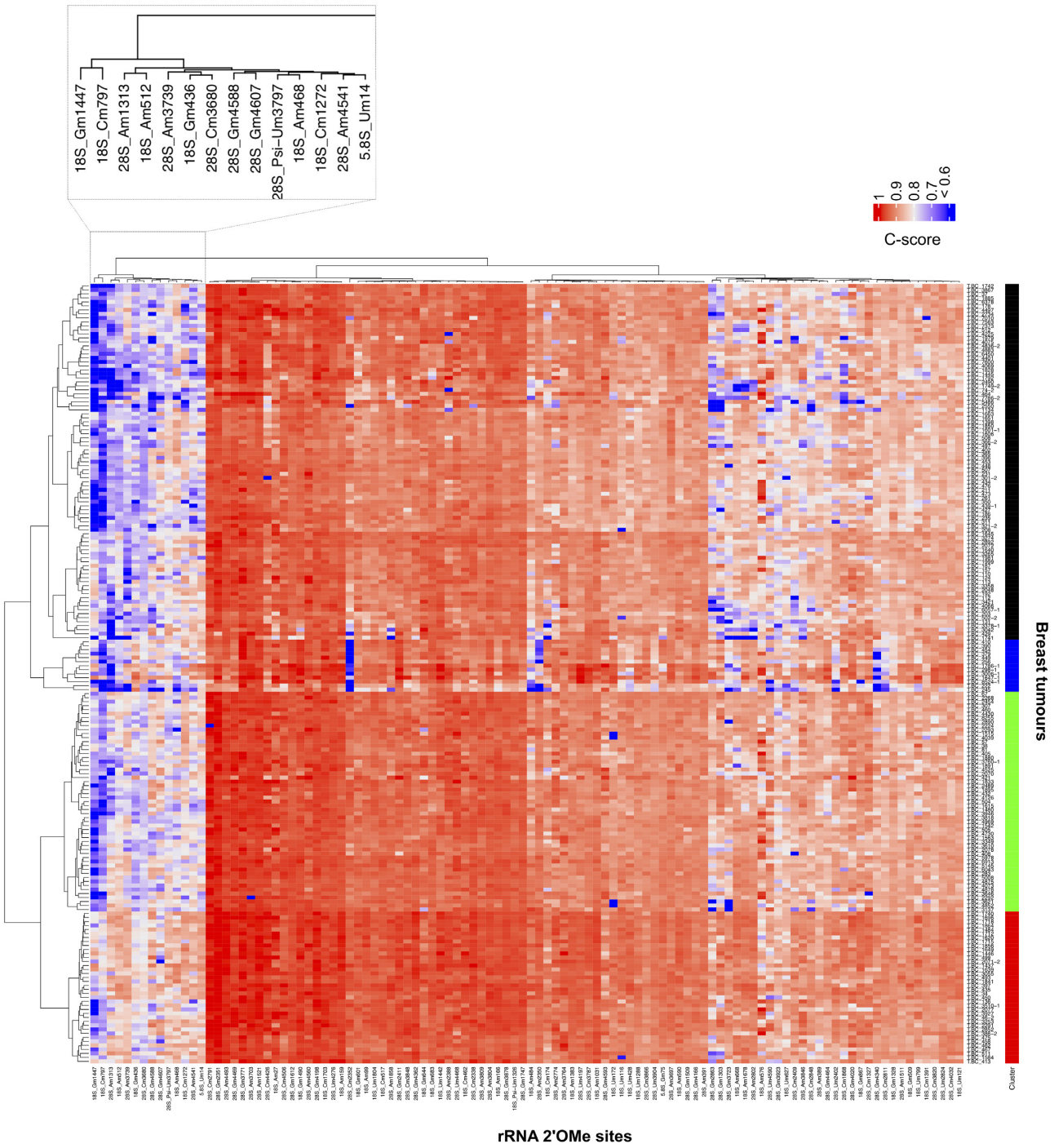


Figure 1. Stability and variability of rRNA 2′O-methylation levels between breast tumours. Levels of rRNA 2′O-methylation (i.e. C-score) were determined at the 106 rRNA 2′O-methylated sites using RiboMeth-seq in a series of 195 primary human breast tumours. Data are presented as a hierarchical clustering where the C-score of each position (column) for each tumour (row) is presented (colour scale). Dendrograms represent relationships of similarity between breast tumours on the basis of their rRNA 2′O-methylation profiles (left panel) that identify four groups of breast tumour samples (right colour panel), or in the rRNA 2′O-methylation level at a given site between tumours (top panel). An enlarged view of a group of clustered rRNA 2′O-methylated sites is presented.

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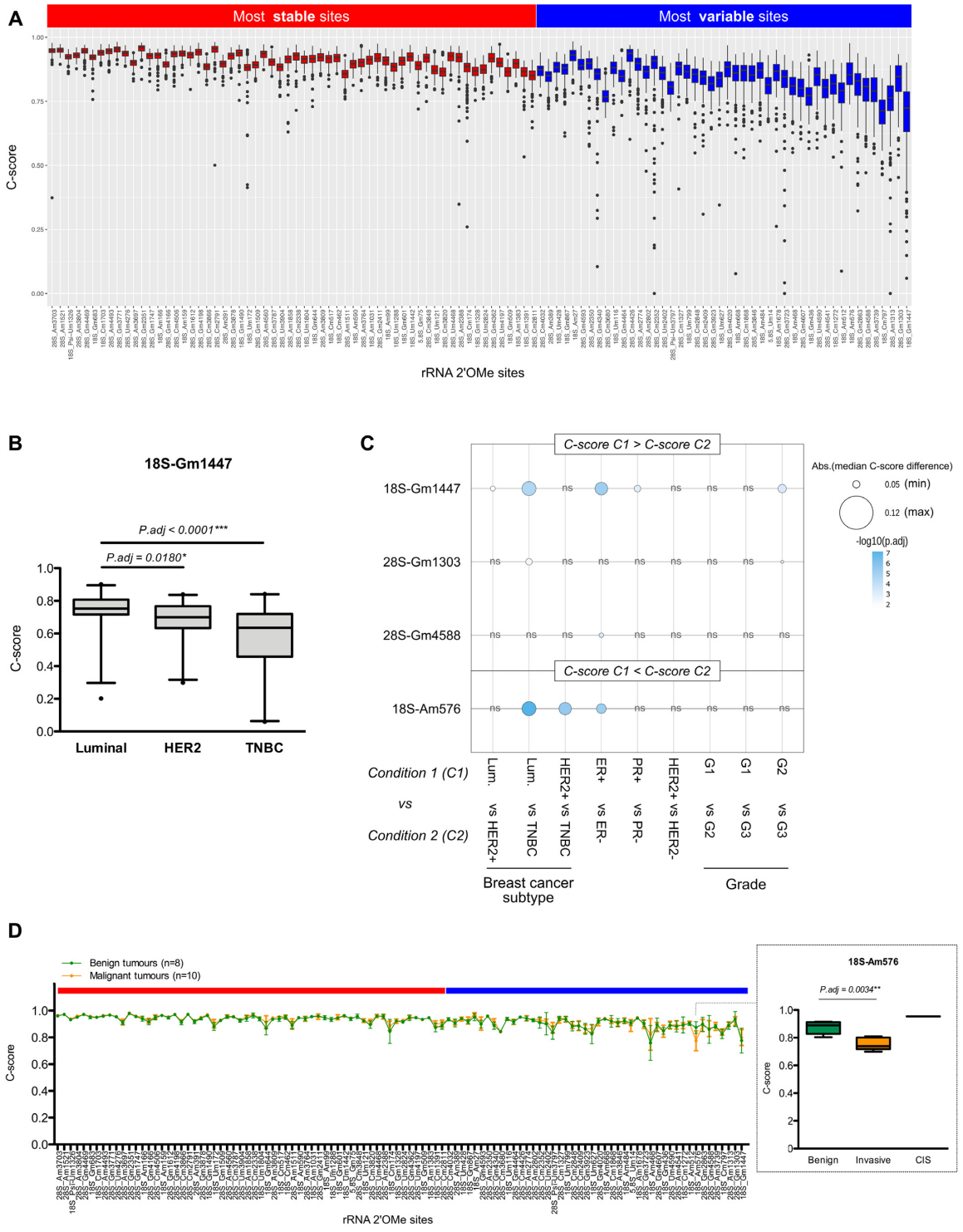


Figure 2. Biological relevance of variable rRNA 2′O-methylation levels in breast cancer. (**A**) C-score variation amongst the 195 human primary breast tumours at each of the 106 rRNA 2′O-methylated sites was ranked by increasing interquartile range (IQR). Based on IQR divergence, two classes of rRNA 2′O-methylated sites were defined: sites with the most stable C-scores (red); and sites with the most variable C-scores (blue). (**B**) Example of significant change in rRNA 2′O-methylation levels at the 18S-Gm1447 site between luminal, HER2+ and TNBC breast tumours. (**C**) Summary of the four variable rRNA sites, whose 2′O-methylation levels were significantly different between breast cancer subtypes, hormonal and HER2 receptors, and tumour grade. ER: oestrogen receptor; PR: progesterone receptor; Luminal: ER+ PR± HER2−; HER2+: ER+/− PR+/− HER2+; TNBC: ER− PR− HER2-; G: grade. (**D**) Comparison of rRNA 2′O-methylation profiles in a series of 8 benign (green) and 10 malignant (orange) tumours. An enlarged view presents the significant change in rRNA 2′O-methylation levels at the variable 18S-Am576 site in malignant compared to benign mammary tumours, with carcinoma in situ (CIS) presenting the highest rRNA 2′O-methylation level. *: adjusted *P*-value < 0.05; **: *P*.adj < 0.01; ***: *P*.adj < 0.0001.

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ability in their 2′O-methylation level ('variable' sites, blue). Amongst them, the 28S-Am1313, 28S-Cm2352 and 28S-Gm3723 sites displayed important inter-patient variability, the C-scores ranging from 0 to >0.90 and a null C-score being observed in different tumours (Figure 2a and Supplementary Figure S3a). Position-by-position comparison of the ∂C-score [(mean C-score + 2sd) – (mean C-score – 2sd)] between the 195 breast tumours and the statistical analysis based on five technical replicates of normal human RNA reference samples reveals that the high inter-patient variability in C-scores corresponds to a biological variability rather than to technical variability (Supplementary Figure S3b). Similarly, these two classes of most stable and most variable sites derived from the 195 breast tumours were observed in a series of 7 mammoplasties derived from healthy female donors, most of the sites being classified in the same classes with the except of few ones (Supplementary Figure S5). The difference might come either from the small size of this series or from the fact that variability in rRNA 2′O-methylation might be tissue-specific, mammoplasties mostly corresponding to adipocyte cells. These data show that rRNA 2′O-methylation varies between human samples and demonstrate the co-existence of stable and variable classes of rRNA 2′O-methylated sites within human samples.

Association of rRNA 2′O-methylation level with biological and clinical characteristics

To assess the biological significance of the co-existence of two classes of rRNA 2′O-methylated positions displaying either the most 'stable' 2′O-methylation level between human tumour samples or the most 'variable' 2′O-methylation level, we evaluated the association between variations in C-score at the 106 rRNA 2′O-methylated positions and biological or clinical characteristics of primary breast tumours (Figure 2B and C). Remarkably, amongst the stable rRNA 2′O-methylated sites, no association between change in C-score and well-known characteristics of breast cancers was observed (i.e. tumour grade, tumour size, lymph node involvement, hormonal/HER2 receptor status, breast cancer subtype or *TP53* mutation). In contrast, we identified four variable rRNA 2′O-methylated sites (18S-Gm1447, 28S-Gm1303, 28S-Gm4588 and 18S-Am576), the levels of which are significantly different between breast cancer subtypes, oestrogen and progesterone statuses, as well as tumour grades (Figure 2B and C; Supplementary Table S3). In both human tumour samples and cell lines, the 2′O-methylation level of the 18S-Gm1447 site decreased in triple-negative breast cancers (TNBCs) compared to the luminal tumours, whilst that of the 18S-Am576 site increased in TNBCs (Figure 2B and C; Supplementary Figure S6). Furthermore, we compared rRNA 2′O-methylation profile in a small series of eight benign (i.e. hyperproliferative, but non-transformed epithelial cells) and 10 malignant tumours, the former do not represent the full breast cancer inter-patient heterogeneity. In this series, 2′O-methylation level at the 18S-Am576 site distinguished benign from malignant mammary tumours (Figure 2D).

Secondly, we evaluated the association between breast cancer patients harbouring tumours exhibiting similar rRNA 2′O-methylation profiles and biological/clinical characteristics of breast tumours. Hierarchical clustering and PCA methods identified 4 clusters of breast cancer tumours based on their rRNA 2′O-methylation profiles (Figures 1 and 3A). Analysis of survival indicates that, although no significant association was observed, the patients carrying breast tumours characterized by distinct rRNA 2′O-methylation profile display distinguishable overall survival (Figure 3B). In particular the 'blue' group displays the poorest overall survival. This blue group was enriched in TNBC and in tumours of high grade compared with the other groups (Figure 3C and D; Supplementary Figure S7). Regarding the fact that tumours in the blue groups exhibited the lowest C-score at most of the rRNA 2′O-methylated sites (Figure 1), these data suggest that global decrease in rRNA 2′O-methylation might be associated with breast tumour aggressiveness. Finally, clustering breast cancer tumours on the basis of their 2′O-methylation level at the 106 rRNA sites highlights at diagnosis, patients carrying small tumours size who display prognosis as poor as patients carrying large tumours size (Figure 3E, blue group). Indeed, patients carrying small tumour size exhibit different survival rates depending on the rRNA 2′O-methylation profile. Overall, these data indicate that 2′O-methylation levels at variable rRNA sites are associated with breast cancer subtypes and tumour grade.

Evaluation of the structural impact of the two classes of rRNA 2′O-methylation sites

To further characterize the two classes of human rRNA 2′O-methylated positions identified in the 195 breast tumours, we compared their conservation during evolution. Amongst the 106 rRNA 2′O-methylation sites described in humans, some sites have also been identified in yeast and bacteria (snoRNA-LBME-db database, www-snorna.biotoul.fr). We thus compared the proportion of either 'stable' or 'variable' sites classified on the basis of our data, amongst three groups: the group of rRNA 2′O-methylated sites only detected in humans ('humans', $n = 68$); the group of rRNA 2′O-methylated sites detected both in humans and yeast ('eukaryotes', $n = 35$); and the group of rRNA 2′O-methylated sites conserved in humans, yeast and bacteria ('universal', $n = 3$; see also annotation on the human ribosome structure in ref 17). Enrichment analysis showed an increased proportion of the variable rRNA 2′O-methylated sites in humans compared to other organisms (Figure 4A), suggesting a link between the appearance of variable rRNA 2′O-methylated sites and evolution, characterized by an increasing number of fine-tuning gene expression mechanisms.

We then mapped the stable and variable rRNA 2′O-methylated positions on the human ribosome structure (16–18). It revealed an interesting distribution in space, where the decoding centre (DC), the peptidyl-transferase centre (PTC) and the polypeptide exit tunnel are mostly decorated with stable 2′O-methylated positions, whilst some variable positions are found in 1 or 2 nt layers away from the functional sites (i.e. maximum 4.5 Å away from the functional sites) (Figure 4B and C, annotated sites). However, 2′O-methylated sites are also found in functional sites, methyl

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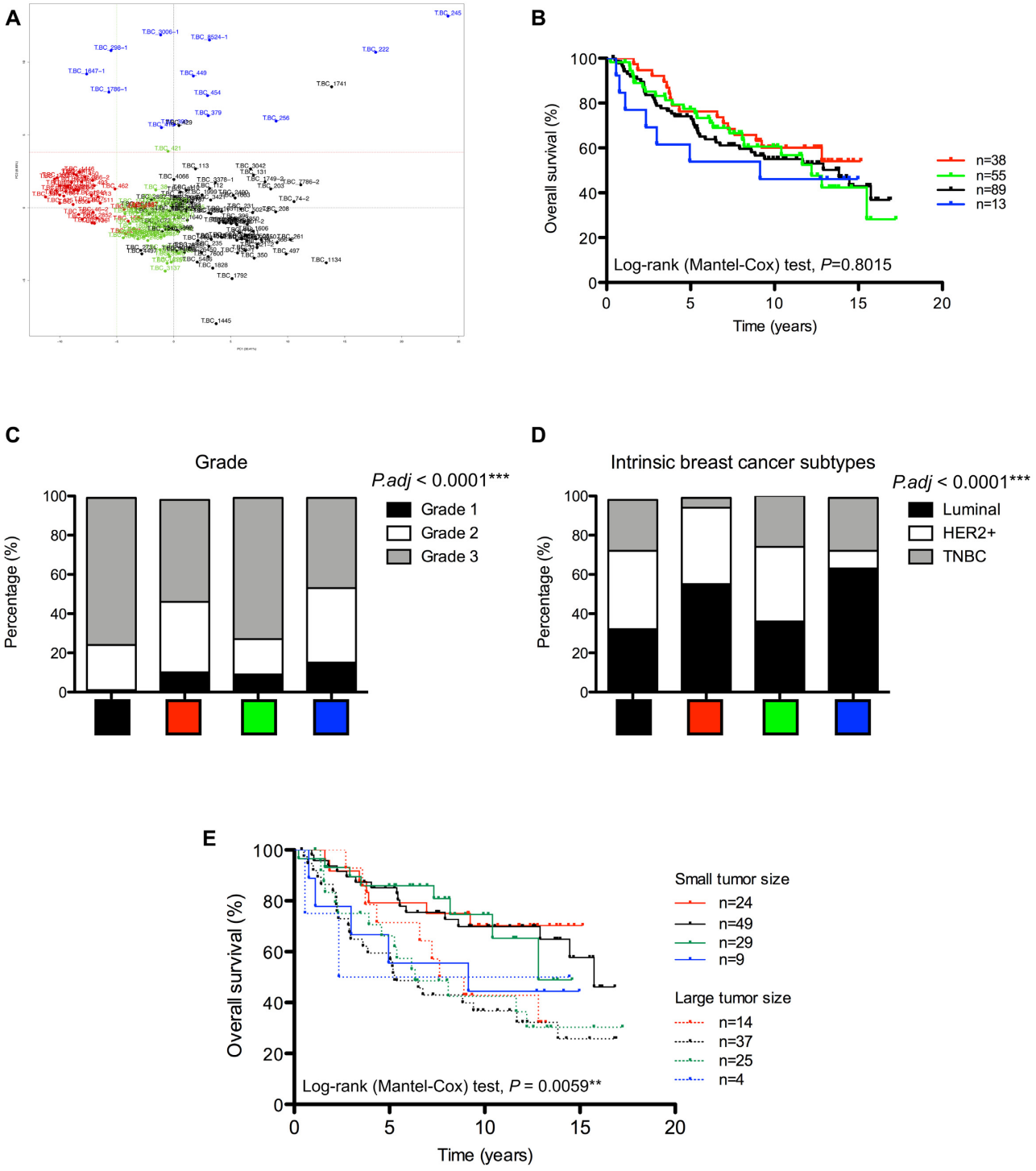


Figure 3. Association of rRNA 2′O-methylation profiles with biological and clinical breast cancer characteristics. (**A**) PCA showed four clusters of breast tumours based on their rRNA 2′O-methylation profiles that correspond to the ones identified using the hierarchical clustering approach (Figure 1). (**B**) Kaplan–Meier curve suggested the patients carrying breast tumours characterized by a 'blue' rRNA 2′O-methylation profile display the poorest overall survival. (**C** and **D**) Significant differences in repartition amongst the four clusters were observed regarding tumour grade (C) and breast cancer subtype (D). Luminal: ER+ PR± HER2−; HER2+: ER+/− PR+/− HER2+; TNBC: ER− PR− HER2−. (**E**) Kaplan–Meier curve depicts the overall survival of patients grouped on the basis of both their tumour size at diagnosis and their rRNA 2′O-methylation profiles. **: $P < 0.01$; ***: $P < 0.0001$.

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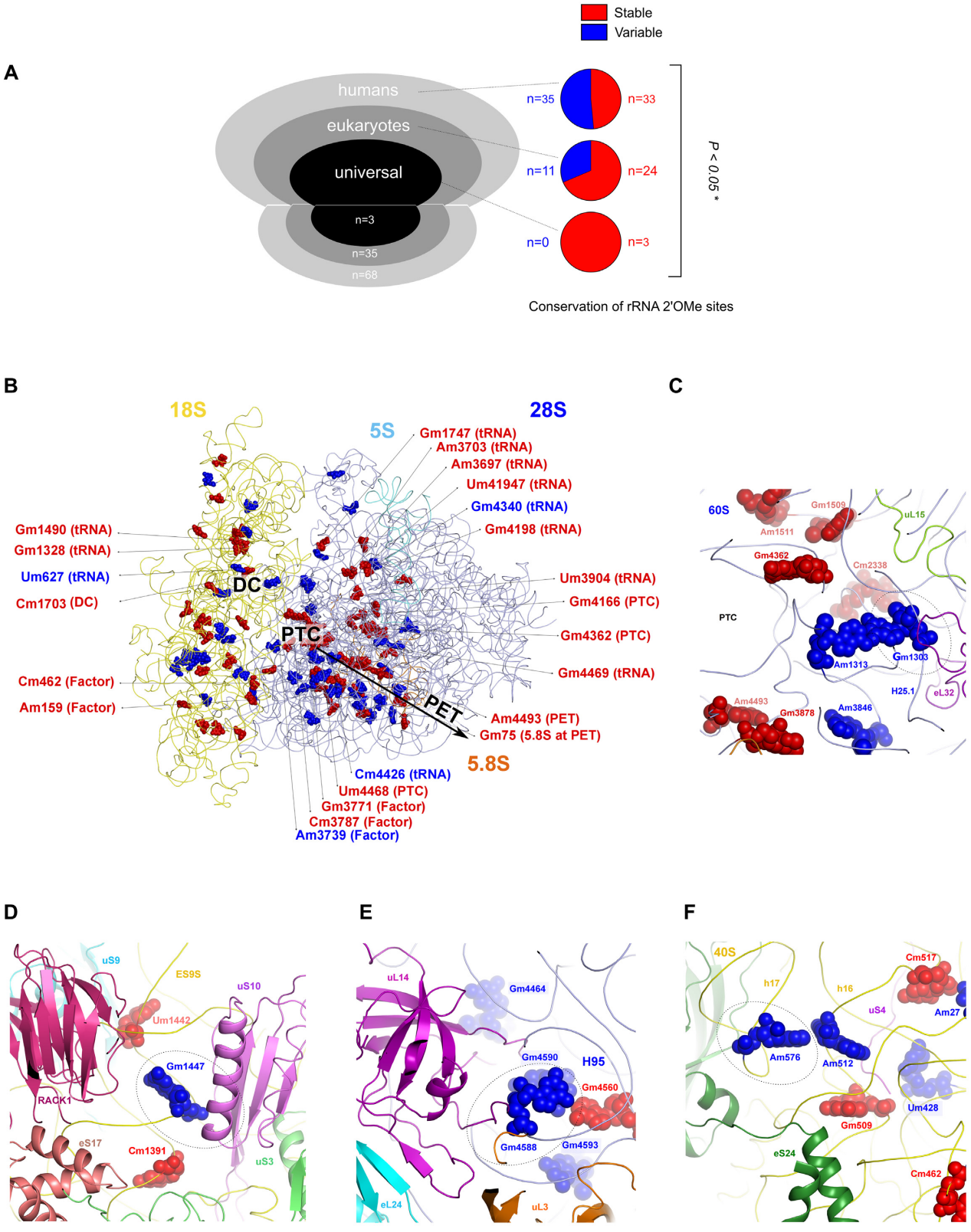


Figure 4. Structural properties of stable and variable rRNA 2′O-methylated sites. (**A**) Amongst the 106 rRNA 2′O-methylated sites in humans, 68 are specific to humans (humans), 35 are found across eukaryotes (eukaryotes) and 3 are conserved from bacteria to humans (universal) (left panel). The proportion of stable and variable sites amongst these three groups is represented using pie charts (right panel). (**B** and **C**), Mapping of the two classes of rRNA 2′O-methylated sites on the human ribosome (B) and at the PTC (C). Only rRNA 2′O-methylated positions directly involved in function domains or in interaction with components of the translational machinery have been annotated. (**D**–**F**) Structural environment of the variable rRNA 2′O-methylated sites significantly associated with breast cancer features. Red: stable sites; blue: variable sites; dotted circles: rRNA 2′O-methylation sites of interest; DC: decoding centre; PTC: peptidyl-transferase centre; PET: peptide exit tunnel. *: $P < 0.05$.

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carbon in very few 2′O-methylated sites are directly involved in van der Waals contacts with tRNAs, mRNA and other ribosome functional large ligands, as can be predicted from modelling these into the human ribosome structure. Most tRNAs binding regions in the large and small subunits are decorated with stable 2′O-methylated positions with the exception of 4 variable 2′O-methylated sites (18S-Um627, 28S-Am3739, 28S-Gm4340 and 28S-Cm4426) (Figure 4B). In particular, the 28S-Am3739 nucleotide is located at the hairpin loop of helix H69, which is located right next to the inter-subunit rotation centre describing pre- and post-translocation rotational states of the 40S with respect to the 60S ribosomal subunit and whose 2′O-methylation may influence P-site tRNA binding. The variable 28S-Gm4340 site is located at the CCA end region of the E-site tRNA that provides favourable van der Waals contacts to the E-site tRNA as observed experimentally in the human 80S structure. Finally, the four rRNA sites displaying changes in 2′O-methylation associated with biological and clinical features of breast cancer (28S-Gm1303, 18S-Gm1447, 28S-Gm4588 and 18S-Am576), are located at the periphery of the PTC of the ribosomes and are accessible for tRNAs, mRNA and other ribosome functional large ligands such as GTPases that associate transiently during the translation process (Figure 4C–F). Various factor binding pockets and their vicinity are mostly decorated with variable sites. Molecular docking tRNA and ribosomal factors suggest that stable rRNA 2′O-methylated sites are mostly located at the catalytic sites of the ribosomes, whilst the variable sites are found at the regulatory sites of the ribosomes.

DISCUSSION

Taken together, we show here for the first time that rRNA molecules exist naturally in humans as molecules not fully and equally 2′O-methylated at all the 106 positions. Indeed, our structure/function and evolution enrichment analyses as well as the differential association with biological characteristics, demonstrates that at least two classes of rRNA 2′O-methylated sites concur in human breast cancers, depending on their ability to tolerate (i.e. variable sites) or not (i.e. stable sites) the absence of chemical modifications. This unique discovery made by comparing human samples demonstrates the co-existence of stable and variable 2′O-methylation at specific rRNA positions.

Historically, plethora of studies mainly performed in both yeast and *Xenopus laevis* models, which have been the historical prototypic, eukaryotic models used during many years finely decipher the highly complex molecular mechanisms of rRNA transcription, chemical modifications and processing (27–31). These studies allowed the mapping of rRNA 2′O-methylation sites but also the demonstration of the importance of 2′O-methylation in the conserved intrinsic activity of the ribosome, on which life relies. Indeed, it appeared over the past years that loss of 2′O-methylation at a few number of rRNA sites in yeast is critical for ribosome functions and induces cell death (27,32). However, more recent findings indicate that in yeast, loss of rRNA 2′O-methylation at other sites have only little or no effect.

Our finding sustain the emerging notion that rRNA chemical modifications provide plasticity to the ribosome structure on which its regulatory effects rely, thus extending the modulation and activity of the ribosome to translational regulation in humans, including in cancer (4,33). In a single human sample, it appears that rRNA 2′O-methylation varies between the 106 rRNA 2′O-methylated positions ($0 < \text{C-score} < 0.98$). It indicates that even within a single human sample, rRNA molecules exist naturally as molecules not fully and equally 2′O-methylated at all the 106 positions. This observation is consistent with previous studies that were performed, albeit in limited number of models, either in cell lines (i.e. HeLa, PA1, HCT-116 and 293) (2,8,18,34–37), in cellular models describing tumour initiation and progression of solid and haematological cancers (1,6,38) or in few number of adult/embryonic organs (39). In addition to these previous studies, our findings also demonstrate that difference in rRNA 2′O-methylation occur between different human samples and reflects inter-patient variability. Variation in rRNA 2′O-methylation level between tumours might result from different mechanisms. Change in 2′O-methylation levels can result from alteration in expression of each individual component of the rRNA 2′O-methylation complex (i.e. the rRNA methyltransferase FBL, the structural proteins Nop56, Nop58 or NHP2L1, or the small nucleolar snoRNA, which hybridizes in a sequence-specific manner the rRNA and thus specifically guides the enzyme) (1,7,9,28). Expression of these different components has been correlated with genomic alteration, loss of tumour suppressor or oncogene activation (1,7,28,40), suggesting that the 106 rRNA 2′O-methylation sites can reflect numerous alterations of the tumour. Moreover, changes in the ratio substrate:enzyme (rRNA:rRNA 2′O-methylation complex) might be sufficient to alter rRNA 2′O-methylation level, the rRNA level being also dependent of particular tumour suppressors and oncogenes (41). Interestingly, the rRNA 2′O-methylated positions that are not fully 2′O-methylated in a single human sample overlap with those displaying strong inter-variability between breast cancer samples. Development of statistical and methodological tools for future meta-analyses will be required to identify the complete list of rRNA sites accepting lack of 2′O-methylation, either commonly or specifically to a particular physio/pathological context.

The diversity of rRNA 2′O-methylation profiles naturally occurring in humans reinforces the altered translational control induced by experimental modulation of rRNA 2′O-methylation (1,2,28). Amongst the variable rRNA 2′O-methylation positions, the 18S-Gm1447 displays the highest variability in rRNA 2′O-methylation amongst tumours with the identification of human tumours and breast cancer cell lines that lack 2′O-methylation at this particular nucleotide. 18S-Gm1447 is in close vicinity of the uS10 ribosomal protein, which has been shown to bind to HCV IRES, regulates translation initiation of short 5′UTR containing mRNAs and contributes in resolving stalled ribosomes (42–46). Thus, absence or presence of rRNA 2′O-methylation at 18S-Gm1447 might finely modulate uS10 activity, which applies to the intrinsic ribosome function in general depending on its plasticity. Overall, our data reinforce, in humans, both the existence of rRNA 2′O-methylated compulsory/stable sites, which display no or low inter-patient variability and on which ribosome activities might rely as suggested by

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structural observations, and of rRNA 2′O-methylated plastic sites, which provides novel properties to the ribosome that can now be seen as a direct actor of translational regulation.

In addition to 2′O-methylation, rRNA exhibits other chemical modifications that affect translational regulation and contribute in the acquisition of cancer hallmarks. rRNA pseudouridylation (Ψ) remains the prototypic example. Alteration in Ψ rRNA profile induced by mutant DKC1, the enzyme-directed rRNA pseudouridine synthesis, has been shown to inhibit translation of the tumour suppressor p53 protein (47,48). More recently, the 18S-U609/U863Ψ has been shown to display tumour suppressive activity in response to KRAS-induced oncogenic stress in liver cancer (49). Similarly, loss of 18S-$m^1acp^3U1248\Psi$ and 28S-m^6A4220 rRNA are associated with colorectal and hepatocellular carcinoma, respectively (50,51). Thus, like other rRNA chemical modifications, rRNA 2′O-methylation can regulate translation and might provide novel molecular understanding of cancer biology, as it has been previously suggested, alteration of rRNA 2′O-methylation profile impairing cell proliferation and resistance to chemotherapy (1). Future studies will thus be required to finely identify rRNA 2′O-methylation sites involved in tumorigenesis by comparing series of normal and tumour breast tissues. Here, using benign tissue corresponding to non-transformed epithelial, we identify that in hyperproliferative cells 2′O-methylation level of the 18S-Am576 site differs between benign and malignant tumours, suggesting that the rRNA 2′O-methylation profile is different between transformed and non-transformed tissue. It has to be noted that due to the cycling evolution of breast cancer through life (i.e. menstruation, pregnancy, menopause…) and the discrepancies between benign tumours (i.e. hyperproliferative), mammoplasty (i.e. astrocyte enrichment) or stromal tissue (i.e. molecular traits) and breast cancer tissues (52), it remains difficult to build matched collection of normal mammary tissues.

Our data also demonstrate that rRNA 2′O-methylation corresponds to an additional layer of molecular characterization of breast cancer patient heterogeneity. Indeed, we show for the first time that rRNA 2′O-methylation levels differ between breast cancer subtypes and tumour grades. rRNA 2′O-methylation corresponds to an additional layer of molecular characterization that may help in the near future to improve breast cancer patient classification but also patients management. First, rRNA 2′O-methylation might be useful to improve the refinement of breast cancer patients stratification required in precision medicine. For example, the rRNA 2′O-methylation profile could help in identifying patients with the poorest outcome although they carry small tumours that are generally associated with a low risk factor in breast cancer. Such patients may thus benefit from a more aggressive treatment protocol usually reserved to treat largest breast tumours. Second, cancer-specific alterations of ribosome structure induced by naturally occurring variation in rRNA 2′O-methylation might be the source of powerful and specific anti-cancer molecules (18,53). Indeed, presence or absence of 2′O-methylation at a particular position might provide formation of specific ligand pockets to specifically target cancer-related ribosomes. In cancer, ribosome indeed appears as an emerging therapeutic target, inhibitors of RNA polymerase I activity specifically killing cancer cells being currently evaluated in clinical trials (54) and small molecules such as eukaryote-specific antibiotics directly targeting the human ribosome reducing proliferation of cancer cells (18,53,55–57). However, future studies will be required to determine whether diversity in rRNA 2′O-methylation profiles result from the intratumoral heterogeneity and/or heterogeneity in chemically modified rRNA molecules between and/or within a single cell.

The discovery of rRNA chemical modifications promoting ribosome plasticity will undoubtedly lead to a deeper and more complete understanding of rRNA chemical modifications in ribosome function and of cell fate determination providing a novel layer of cancer-related molecular fingerprinting.

DATA AVAILABILITY

Deposit FastQ and/or raw counts as dataset in GEO Profile (GSE143415).

To review GEO accession GSE143415:

Go to https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE143415.

Enter token wpknugomppoxlmh into the box.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Cancer Online.

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