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RAPID COMMUNICATION

m^6A levels and expression of its modification genes show significant differences in breast cancer molecular subtypes

N^6-methyladenosine (m^6A) is the most abundant modification in eukaryotic messenger RNA.[1] This modification is dynamic, reversible, and mediated by proteins characterized as methyltransferases and demethylases. Recent investigations have found that aberrant expression of methyltransferases and demethylases results in m^6A dysregulation and, in consequence, affects the biological functions in which this modification is involved. Indeed, m^6A dysregulation affects the development and maintenance of various diseases, including cancer.[2] For this reason, we explored the potential role that m^6A modification has in invasive breast cancer of no special type (IBC-NST) and its molecular subtypes luminal A, luminal B HER2-, luminal B HER2+, HER2+, and triple-negative breast cancer (TNBC) whose degree of global m^6A methylation has not yet been studied.

Materials and methods for the present study are available in full in the Supplementary Material. Briefly, we obtained total RNA from frozen tumors and healthy tissue samples from 35 patients with IBC-NST classified into molecular subtypes. Subsequently, we used the obtained RNA to estimate the global m^6A methylation percentage as well as the gene expression of methyltransferases (*METTL3*, *METTL14*, and *WTAP*) and demethylases (*FTO* and *ALKBH5*) and their correlations in tumor and healthy tissues.

Using a global m^6A colorimetric quantification method, we observed a percentage of methylation significantly decreased in tumor tissues compared to healthy breast tissues (0.37 ± 0.09 in tumor and 0.47 ± 0.08 in healthy tissue samples of the patients, $P = 0.001$) (Fig. S1A). Consequently, we evaluated the expression of the main genes involved in methylation and demethylation to determine their possible dysregulations as the cause of this decrease. We found that gene expression of analyzed methyltransferases (*METTL3*, *METTL14*, and *WTAP*) and demethylases (*FTO* and *ALKBH5*) was dysregulated in tumor samples and, in all cases, their expression was significantly lower than in healthy tissues (Table S3 and Fig. S2). Therefore, we infer that the low percentage of global m^6A methylation found in tumor samples could be mainly due to the lack of methylation by the methyltransferases complex which the reduction in the expression levels of the genes that code for the demethylases does not compensate.

As explained in the Supplementary Discussion of the Supplementary Material, the regulation of the main methyltransferase and demethylase enzymes is not fully understood but it seems that they may be subject to different regulation mechanisms, including but not limited to epigenetic modifications. As for their gene expression, previous studies have reported mixed results in breast cancer as summarized in Table S4. We consider that possible reasons for these differences in the expression of m^6A modification genes include differences in the number and type of samples analyzed in the studies, the method used to determine gene expression, and the fact that the samples were analyzed without taking into account their molecular subtype. Therefore, we analyzed our results considering the different molecular subtypes. We found that all the subtypes coincided in a low m^6A methylation percentage in the tumor samples (0.43%–0.54% for healthy tissues and 0.28%–0.42% for tumor tissues), although we only detected significant differences between tumor samples and their corresponding healthy tissues in the luminal A subtype ($P = 0.04$) (Table S5 and Fig. S1B–F). When we analyzed the expression of the methyltransferase and

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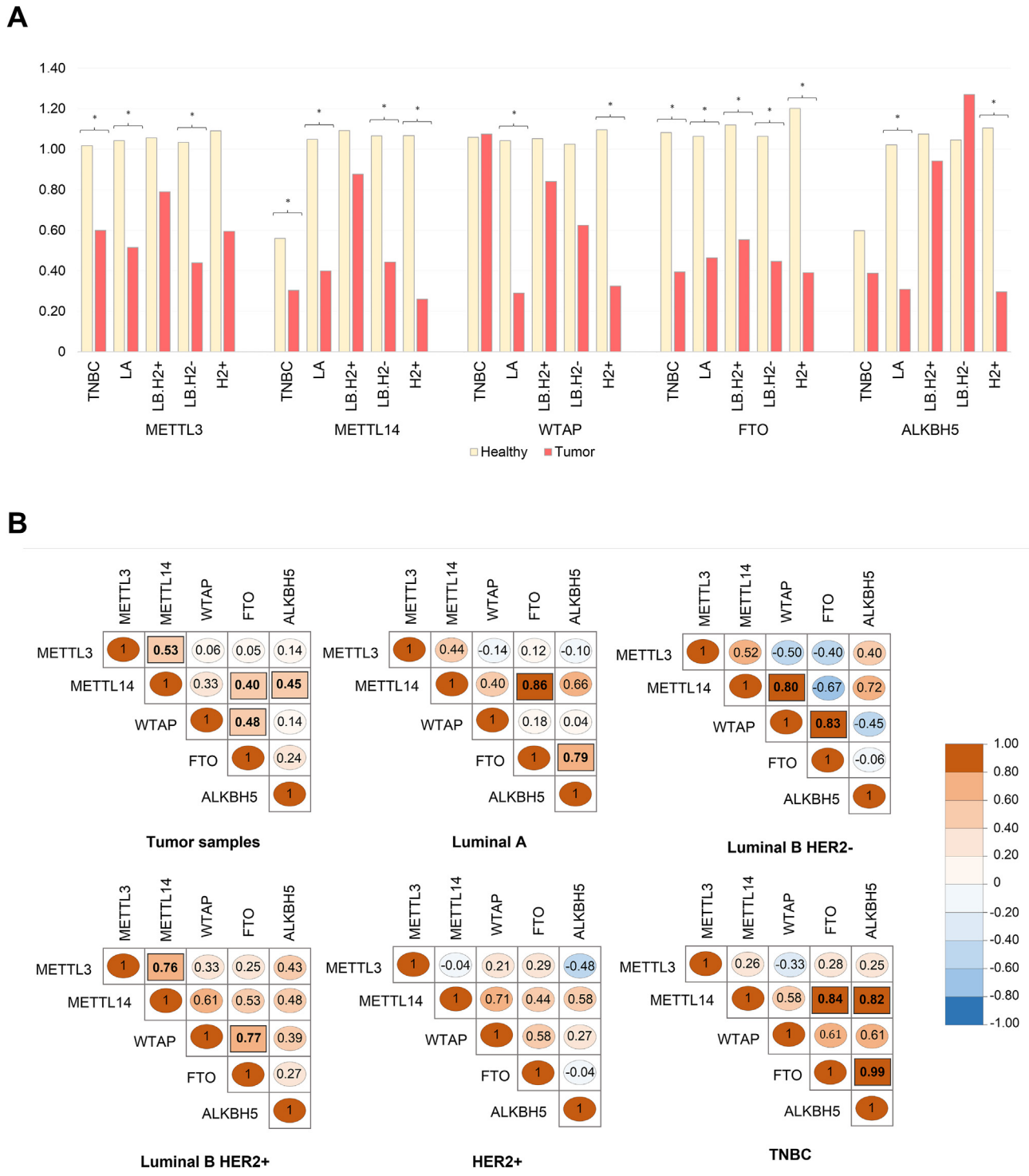

Figure 1 Main results of gene expression and correlation analysis. **(A)** Summary of the expression levels for *METTL3*, *METTL14*, *WTAP*, *FTO*, and *ALKBH5* genes obtained for the different molecular subtypes for tumor and their corresponding healthy breast tissues. *$P < 0.05$. The Kruskal–Wallis *H* test showed significant differences between subtypes for *METTL14*, *WTAP*, and *ALKBH5* (see Table S6 for numerical values). **(B)** Correlation analysis of the gene expression levels of *METTL3*, *METTL14*, *WTAP*, *FTO*, and *ALKBH5* in the tumor samples of the 35 IBC-NST patients. This test was also carried out taking into account the different molecular subtypes. The significant correlations were indicated in bold and in the box. *P* values < 0.05 were considered statistically significant.

demethylase genes in the five subtypes, they varied from one to another (Table S6 and Fig. S3—S4). However, in all significant cases, compared with the healthy breast tissues, the expression in tumor tissues was reduced. Figure 1A presents a summary of the results obtained in the gene expression analysis. *FTO* expression was significantly low in tumor samples in all analyzed breast cancer subtypes, which we might expect to increase the presence of m^6A. However, our results show a general reduction of m^6A in tumor samples when compared to healthy tissue. A possible explanation could be the limited effect of FTO on RNA demethylation. Mauer and Jaffrey[3] have argued that FTO does not have substrate-specific activity for m^6A because its demethylation rate toward m^6A is slow (as opposed to the rate of demethylation of m^6Am) and its role as an m^6A demethylase has come into question. Indeed, in some studies, no increases in m^6A methylation levels were detected in mRNA when using an *FTO* knockdown or knockout tissue.[1] These considerations would explain why samples with reduced methyltransferase gene expression would have lower m^6A levels even if *FTO* expression is also reduced. On the other hand, the methylation of m^6A in mRNA occurs as a result of the methyltransferase complex composed of proteins METTL3, METTL14, and WTAP. Although METTL3 is the only protein in the complex with methyltransferase activity,[4] the other two are necessary for the methylation reaction. In particular, WTAP works as an adaptor protein without catalytic activity and ensures the presence of METTL3-METTL14 associated with nuclear speckles.[5] In the absence of *WTAP* mRNA methylation decreases dramatically because this protein influences the binding of the catalytic part of the methyltransferase complex (METTL3) to the RNA.[1] Thus, the reduction of *WTAP* could explain the reduction in methylation in samples with low ALKBH5 and FTO activity (from HER2+ and luminal A subtypes), even though studies showed that reduced ALKBH5 activity could lead to higher m^6A levels.[1]

We also assessed whether there were significant differences between tumor samples of the different subtypes by the Kruskal—Wallis H test (Table S6 and Fig. S5). The expression of *METTL3* and *FTO* were similar in all subtypes and did not show significant differences. On the contrary, the expression of *METTL14*, *WTAP*, and *ALKBH5* genes presented differences between some subtypes as shown in Table S7. Finally, since the methyltransferases and demethylases are all involved in m^6A modification, we evaluated whether there was any correlation at the gene expression level. As shown in Figure 1B, the most frequent correlations occur between methyltransferases and demethylases, except in the HER2+ subtype. We found different specific statistical correlations for each molecular subtype. Thus, these results further support the need for epigenetic characterization of different molecular subtypes.

In summary, current results indicate that despite being correlated, the reduction in the expression of demethylase genes in IBC-NST does not cancel out the reduction in methyltransferase expression. As a result, we find a net demethylation of m^6A. Besides, each molecular subtype presented a characteristic gene expression profile of methyltransferases and demethylases as well as correlations between them, which could be useful for molecular tumor classification. These results accentuate the need to analyze each subtype in depth. With these findings, we will understand better the modulation of m^6A methylation and its implication in the development of tumorigenesis in different molecular subtypes of IBC-NST.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.11.015.

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