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**mRNA and miRNA expression profiling of Follicular Variant of Papillary Thyroid
Carcinoma with and without distant metastases**

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1 Abstract

2 Follicular Variant of Papillary Thyroid Carcinoma (FVPTC) is usually associated with a good outcome.
3 Nevertheless, in rare cases, it develops distant metastases (1-9%).

4 Our goal was to investigate whether mRNA and miRNA expression profiles may help distinguish between
5 metastatic versus non-metastatic FVPTCs.

6 Twenty-four primary FVPTCs, 12 metastatic and 12 non-metastatic, with similar clinicopathological features
7 were selected and analyzed by nanoString nCounter technology using two distinct panels for expression
8 analysis of 740 mRNA and 798 miRNAs. Data analysis was performed using the nanoString nSolver 3.0
9 software.

10 Forty-seven mRNA and 35 miRNAs were differentially expressed between the two groups. Using these
11 mRNA and miRNAs, metastatic and non-metastatic FVPTCs were clearly divided into two distinct clusters.

12 Our results indicate that FVPTCs with metastatic abilities have different expression profiles compared to the
13 non-metastatic. A prospective validation is needed to evaluate the usefulness of this molecular approach in
14 the early identification of high-risk FVPTCs.

15

16 *Keywords:* FVPTC; Distant Metastasis; mRNA Expression, miRNA Expression.

17 **Introduction**

18 The incidence of thyroid cancer and the mortality rate have increased over the past few decades worldwide (
19 Cancer Facts and Figures 2018).

20 Despite the advances in knowledge and treatment of thyroid carcinoma, metastases continue to be the most
21 significant cause of thyroid cancer mortality (Kitamura et al., 1999; Mazzaferri and Kloos, 2001).

22 Follicular variant of papillary thyroid carcinoma (FVPTC) is one of the most frequent and fastest growing
23 subsets of papillary thyroid carcinoma (PTC), accounting for 15 to 30% of all PTC cases. FVPTCs can be
24 divided into two distinct subtypes: the encapsulated forms with or without capsular and/or vascular invasion
25 and the non-encapsulated or infiltrative forms with invasion of the surrounding thyroid parenchyma (Liu et
26 al., 2006; Piana et al., 2010; LiVolsi, 2011; Kakudo et al., 2012; Vivero et al., 2013). Recently, several
27 studies have attempted to analyze the behavior and outcome of FVPTC. All of those works concurred that
28 FVPTC display an intermediate behavior and clinicopathological features between those of the classical
29 variant (CV) of PTC and those of the follicular thyroid carcinoma (FTC), making it challenging to establish a
30 standard treatment protocol (Yu et al.,2013; YANG et al., 2015).

31 Usually, FVPTC is associated with a good outcome, especially if the tumor is encapsulated without capsular
32 and/or vascular invasion. On the contrary, the encapsulated form of FVPTC with capsular and/or vascular
33 invasion and the non-encapsulated form show a more aggressive behavior (Liu et al., 2006; LiVolsi and
34 Baloch, 2009; Rivera et al., 2010; Vivero et al., 2013; Tallini et al.,2016).

35 Several studies have shown that the age at diagnosis, histology, tumor size, extrathyroidal extension, lymph
36 node and distant metastases are predictors of prognosis in thyroid cancer (Nixon et al., 2012; Podnos et al.,
37 2005; Sampson et al., 2007; Shah et al., 1992; Zhao et al., 2012).

38 Distant metastasis (DM) affects 3-20% of patients with differentiated thyroid cancer, and up to 50% of these
39 patients show a metastatic disease at diagnosis or during the first few months after surgery (Shaha et al.,
40 1997; Lin et al., 1999; Benbassat et al., 2006; Mihailovic et al., 2007; Lee and Soh, 2010; Schneider et al.,
41 2015; Farina et al., 2016).

42 The frequency of DM is different among the various thyroid histotypes (Pomorski and Bartos, 1999;
43 Ruegemer et al., 1988; Shaha et al., 1996; Haq and Harmer, 2005). Regarding FVPTCs, DM are uncommon
44 and affect only 1% to 9% of the patients (Li et al., 2016; Schneider et al., 2015; Shaha et al., 1997; Shi et al.,

2016; Xu et al., 2017). In those rare cases, the most common site of metastasis are the lungs followed by the bones. Other metastatic areas are less common and involve the mediastinum, brain, liver and skin (Nwaeze et al., 2015; Daniels, 2016).

Higher rates of distant metastases from FVPTCs are described in patients with non-encapsulated forms, extrathyroidal extension, and angiolymphatic invasion (Haq and Harmer, 2005; Li et al., 2016). In particular, some authors reported that 25 to 50% of FVPTCs with vascular invasion show distant metastases (Mete and Asa, 2011).

The clinicopathological features of a tumor by themselves are not sufficient to predict the development of a distant metastatic disease in FVPTCs.

Important questions about metastatic progression remain to be addressed. Currently, the main challenge is understanding metastatic potential at the molecular level.

In this regard, several studies have reported comparisons of the gene expression profiles between primary and metastatic tumors, such as melanoma, breast, colon and lung cancer (Ganepola et al., 2010; Ramaswamy et al., 2003; Weigelt et al., 2003).

Furthermore, numerous studies demonstrated that microRNAs (miRNA) play a key role in metastatic progression. These miRNAs control metastatic potential through the divergent or convergent regulation of metastatic gene pathways (Baranwal and Alahari, 2010; Pencheva and Tavazoie, 2013; Seven et al., 2014).

However, unlike other tumor models, very few studies concern the identification of peculiar molecular profiles for the metastatic potential of thyroid cancer exist (Londero et al., 2016). More importantly there are no studies investigating the presence of a molecular signature for the metastatic potential of FVPTCs.

Therefore, according to this literature data, we aimed to investigate the molecular basis underlying the different ability of FVPTCs to metastasize with similar histopathological characteristics. In this study, we characterized the molecular landscape of metastatic and non-metastatic FVPTC by analyzing two distinct panels that consist of carefully selected mRNAs and miRNAs.

Materials and Methods

Patients and Study Design Between 2000 and 2014, 14,082 consecutive patients underwent thyroid surgery at the Department of Surgical, Medical, Molecular Pathology and Critical Area of the University of Pisa and

72 had a diagnosis of papillary thyroid carcinoma. Among them, 4,847 (34.4%) were FVPTCs. Of the 4,847
73 FVPTC patients, 47 (0.9%) patients developed DM beyond the locoregional neck area (figure 1).

74 We conducted this study matching a group of 12 primary metastatic (Met) FVPTCs with 12 non-metastatic
75 (NonMet) FVPTCs fulfilling all the following inclusion criteria: 1) absence of solid/trabecular areas of more
76 than 30%, 2) absence of papillary growth, 3) absence of insular areas, 4) absence of necrosis, 5) absence of
77 mitotic activity $>3/10\text{HPF}$, 6) in the presence of multifocality, the tumor with the greatest size or highest pT
78 status was always analyzed, and 7) absence of poorly differentiated areas in histologically proven distant
79 metastasis.

80 The NonMet group was made up by searching for FVPTCs with clinicopathological features similar to those
81 of the Met group, including age, gender, tumor size and pathological staging (figure 2). To exclude the
82 biochemical persistence of the disease or the developing of occult loco-regional recurrence, nodes or distant
83 metastases, we chose NonMet patients with a mean follow-up of 9 years.

84 Histological diagnoses were reviewed in a blinded fashion by three pathologists (F.B , L.T, C.S.) according
85 to the World Health Organization criteria (Lloyd, 2017). Tumors were staged according to the 8th edition of
86 the tumor node-metastasis-based staging system recommended by the American Joint Commission on
87 Cancer (AJCC) and the International Union Against Cancer (Amin, 2017).

88 After the initial review and case selection, fourteen clinicopathological features (age, gender, tumor size,
89 histological subtype, capsular invasion, vascular invasion, extrathyroidal invasion, intra-tumoral fibrosis,
90 multifocality, bilaterality, stage, distant metastasis, years of follow-up and disease status at the last follow-
91 up) were evaluated for each case for the two groups.

92 After surgical treatment, all the patients were followed at the Department of Clinical and Experimental
93 Medicine of University of Pisa. When indicated, they were treated with low radioiodine (^{131}I) activities for
94 postsurgical thyroid remnant ablation. Subsequent treatments of ^{131}I were administered when required.

95 At 12 months, the patients underwent physical examination, neck ultrasound (US) evaluation, recombinant
96 human TSH stimulation tests for serum thyroglobulin (sTg) or basal ultrasensitive Tg measurement, and in
97 cases of detectable anti-Tg antibodies (TgAb), a diagnostic whole-body scan (dWBS).

98 Patients were considered to be free of disease when their sTg levels after recombinant human TSH were less
99 than 1 ng/mL or their basal ultrasensitive Tg was less than 0,1 ng/mL, neck US was negative, and TgAb was

100 undetectable. The patients who did not undergo ^{131}I remnant ablation were considered to be free of disease
101 when the neck US was negative and sTg and TgAb were undetectable and/or stable during follow-up.
102 Patients free of disease were followed up every 12–18 months. Patients who were not considered free of
103 disease underwent subsequent ^{131}I and/or other surgical treatments if necessary. Moreover, in case of
104 suspicious distant metastases, other imaging studies such as computed tomography, Positron Emission
105 Tomography, Magnetic Resonance Tomography and bone scintigraphy were performed.

106 **RNA and miRNA extraction** For each sample, four FFPE tissue sections, with a thickness of 5 μm ,
107 underwent standard deparaffinization and enrichment by manual microdissection. The last section was
108 stained with hematoxylin and eosin; the tumor area was marked, and the percentage of tumor cells was
109 estimated by a pathologist. The mean proportion of tumor cells in the analysed samples was at least 70%.
110 The tumor tissue was manually microdissected to obtain tumor cell enrichment by excluding lymphocytic
111 infiltration, hemorrhagic area, and fibrous tissue. Total RNA, including miRNAs, were isolated using a
112 commercial kit (miRNeasy Mini Kit, Qiagen, Hilden, Germany) according to the manufacturer's
113 instructions. The RNA concentration was assessed using a spectrophotometer (Dropsense Xpose, Trinean,
114 Gentbrugge, Belgium). The RNA resulted adequate for mRNA expression whenever its concentration was \geq
115 30 ng/ μL and its quality was acceptable if the ratio between the value of the absorbance (A) at 260 nm and
116 the absorbance at 280 nm was ≥ 1.8 , and the ratio between the value of absorbance (A) at 260 nm and the one
117 at 230 nm was ≥ 2 .

118 **NanoString nCounter Assay** The nCounter PanCancer Progression Panel and the nCounter v3 miRNA
119 assay Panel used in this study were designed and synthesized by NanoString Technologies (NanoString,
120 Seattle, WA, USA).

121 For the mRNA expression, the panel was created by cross referencing several public databases and consisted
122 of 740 endogenous human genes involved in 4 major processes of tumor progression: angiogenesis (ANG),
123 extracellular matrix (ECM), epithelial-mesenchymal transition (EMT) and metastasis (META), plus 30
124 housekeeping genes for reference.

125 For the miRNA expression, the panel included unique oligo-nucleotide tags onto 798 highly curated human
126 miRNAs (from miRBase v21) and five housekeeping mRNAs for reference (*ACTB*, *B2M*, *GAPDH*, *RPL19*

127 and *RPLP0*). Twenty-five control probes recognizing either synthetic mRNA or miRNA targets were used
128 to monitor the efficiency and specificity of each reaction step.

129 Each sample was analyzed with both panels by using 150 ng of total RNA for hybridization (21 h at 65°) in
130 addition to the probe pairs consisting of a Reporter Probe, which carry the signal on their 5' end, and a
131 Capture Probes, which carries biotin on their 3' end. After hybridization, sample cleanup and digital report
132 counts were performed according to the manufacturer's instructions.

133 **Data Normalization** mRNA expression data and miRNA expression data were analyzed using nanoString
134 nSolver version 3.0 software.

135 For mRNA expression analysis, raw data normalization was executed in two steps. The first was a technical
136 normalization. The background noise was subtracted using 8 negative controls. The mean and standard
137 deviation (SD) were calculated from the negative controls, and the threshold was defined as the mean plus 2
138 SD. Furthermore, a normalization factor was calculated by obtaining the geometric mean of the six internal
139 positive controls used for each sample to remove the potential systematic differences between the individual
140 hybridization experiments. For each sample, a positive control scaling factor was calculated. If the calculated
141 positive control scaling factor was outside a range of 0.3-3, it indicated technical problems, implicating the
142 exclusion of the sample from further analysis. On the other hand, the second was a biological normalization
143 using housekeeping genes to remove any effect that might be attributed to differences in the amount of input
144 RNA. In detail, this normalization corrects for differences in the RNA input among the assays, allowing the
145 adjustment of gene counts on the basis of the reference genes. For each sample a biological normalization
146 factor was determined and whenever it was outside the range of 0.1-10.0, the sample was excluded from the
147 analysis. All the normalization steps were performed according to the manufacturers' instructions.

148 For miRNA expression, we calculated a background level of expression for each sample using the mean
149 level of the negative controls plus two SD. MiRNAs expressed less than two standard deviations from the
150 mean and were excluded from further analysis. After that, miRNA input levels were normalized using the
151 geometric mean of the top 100 miRNAs with lower variability coefficients, according to the manufacturer's
152 protocol. All the normalization steps were performed according to the manufacturers' instructions.

153 **DIANA-miRPath v3.0** DNA Intelligent Analysis (DIANA) - miRPath v3.0, a web-based computational
154 tool, was used to evaluate the potential interactions between the differentially expressed miRNAs and

155 mRNAs. This software predicts the miRNA targets with high accuracy based on the experimentally
156 supported miRNA-mRNA interactions from TarBase, v7.0. It performs an enrichment analysis to identify the
157 target genes of multiple miRNAs in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways
158 (Vlachos et al., 2015). As our analysis is hypothesis-free, we used the “pathway union” option of miRPath
159 software. *P*-values were obtained by Fisher’s exact test as enrichment analysis method and the false
160 discovery rate (FDR) was estimated using the Benjamini and Hochberg method (Benjamini and Hochberg,
161 1995).

162 **Statistical Analysis** The differential mRNAs and miRNA expression between the metastatic and non-
163 metastatic FVPTC samples was tested using the Mann-Whitney U test followed by the Benjamini-Hochberg
164 correction.

165 Given the exploratory nature of the present study to detect differential expressed mRNAs and miRNAs to be
166 validated in future experiments, a liberal cutoff of 0.25 for the FDR was set as the significant threshold.

167 Statistical analysis was performed using R software package, version 3.4.0.

168 Hierarchical clustering was performed with the NanoString nSolver version 3.0 software on normalized data
169 using Pearson correlation (*r*).

170 The association between the differentially expressed miRNAs and the differentially expressed mRNAs was
171 carried out by (DIANA)-miRPath v3.0 software using $FDR < 0.05$ as the significance threshold (Vlachos et
172 al., 2015).

173 **Results**

174 **Clinicopathological features of FVPTCs with and without distant Metastases** Clinicopathological
175 parameters of all the cases are reported in Table 1. No significant differences regarding age, gender, tumor
176 size, histological subtype, capsular invasion, vascular invasion, extrathyroidal invasion, intra-tumoral
177 fibrosis, multifocality, bilaterality and pT stage have been observed between the Met and NonMet groups.

178 None of the cases both in Met and NonMet groups presented lymph-node metastases. In details, one out of
179 12 Met patients underwent to lymphadenectomy, resulted negative for metastasis at histological examination.

180 The mean follow up was 5.1 years for the Met tumors, while patients without metastasis have been observed
181 for 9.3 years.

182 Twenty-one out of the 24 cases were encapsulated FVPTCs with capsular invasion (11 Met, 10 NonMet) and
183 three out of 24 were infiltrative FVPTCs (1 Met, 2 NonMet). The vascular invasion foci were observed in 8
184 Met FVPTCs (6 with <4 foci, and 2 with ≥ 4 foci) and 5 NonMet FVPTCs (3 with <4 foci, and 2 with ≥ 4
185 foci).

186 NonMet patients showed negative neck US evaluation and undetectable sTg and TgAb levels at their last
187 screening visit.

188 Of the 12 Met patients, 4 developed DM before surgery, 3 patients within 12 months after thyroidectomy,
189 and 5 patients with DM were found during the follow-up period.

190 Metastatic lesions have been confirmed histologically in 10 out of 12 patients. Nine patients had a single DM
191 located in bone (6), central nervous system (2) and lung (1); one patient developed multiple bone metastases.

192 Two patients had a DM detected in first ^{131}I WBS (multiple bone and thorax uptake).

193 **mRNA Expression Profile of Metastatic and Non-metastatic FVPTCs** One of samples failed the
194 biological normalization, thus indicating an mRNA input of poor quality, and was excluded from further
195 analyses. The expression profile of the 740 mRNAs was carried out on 23 tumor samples, including 12
196 (52%) Met FVPTCs and 11 (48%) NonMet FVPTCs, to identify differentially expressed mRNAs between
197 the two groups.

198 Setting an $\text{FDR} < 0.25$, 47 out of 740 mRNAs were differentially expressed between the Met and NonMet
199 lesions. In detail, a total of 4 mRNAs (*SMC3*, *TOM1L1*, *PLA2G2A* and *F11R*) were upregulated in Met
200 FVPTCs, whereas 43 mRNAs were downregulated (Table 2).

201 **Association between Differentially Expressed mRNAs and tumor progression pathways** The
202 differentially expressed mRNAs in the Met versus NonMet FVPTCs were implicated in different pathways,
203 as shown in Table 2. In detail, the 4 mRNAs upregulated in the Met group were: *SMC3* (Structural
204 Maintenance Of Chromosomes 3), which is involved in remodeling of basement membrane (ECM process)
205 and tumor growth, *TOM1L1* (target of myb1 (chicken)-like 1), which is implicated in the Epithelial-
206 Mesenchymal Transition (EMT process), *PLA2G2A* (phospholipase A2, group IIA), which is related to
207 VEGFA signaling (ANG) and *F11R* (F11 receptor), which is associated with Blood Coagulation (ANG
208 process), Cell Adhesion and Cellular Differentiation (EMT process), remodeling of the Integral to Membrane
209 and Plasma Membrane structural components (ECM process).

210 On the contrary, among the 43 downregulated mRNAs in the Met versus NonMet tumors, 27 mRNAs
211 (62.8%) were involved in ANG, 14 mRNAs (32.5%) in EMT, 13 mRNAs (30.2%) in ECM and 6 mRNAs
212 (13.9%) in META. Moreover, 15 of these downregulated mRNAs were involved simultaneously in different
213 pathways.

214 **Cluster Analysis using 47 Differentially Expressed mRNAs** To compare the mRNAs expression profile
215 between the Met and NonMet FVPTCs, an unsupervised hierarchical clustering with a Pearson correlation
216 was performed using the 47 differentially expressed mRNAs.

217 Two distinct clusters of tumors were observed in the dendrogram showed in figure 3. The first cluster
218 included 9 out of 11 (82%) Met FVPTCs, whereas the second one consisted of 9 out of 12 (75%) NonMet
219 FVPTCs. This approach suggests that the tumors can be divided into two types based on this set of 47
220 significant mRNAs.

221 **miRNA Expression Profile of Metastatic and Non-metastatic FVPTCs** Two samples were excluded from
222 further analysis on the basis of the biological normalization factor, thus indicating an mRNA input of poor
223 quality. The expression profiling of 798 miRNAs was evaluated on 22 tumor samples, including 11 (50%)
224 Met FVPTCs and 11 (50%) NonMet FVPTCs.

225 Six-hundred-twenty-one miRNAs with an average count of less than the mean plus 2 SD of the negative
226 controls were excluded.

227 As reported in Table 3, setting an $FDR < 0.25$, 35 out of the 798 miRNAs were differentially expressed
228 between the two groups. Fifteen were upregulated in the Met group, whereas 20 were downregulated.

229 **Cluster Analysis using 35 Differentially Expressed miRNAs** An unsupervised hierarchical clustering
230 according to the 35 differentially expressed miRNAs was performed using nSolver Analysis software with a
231 Pearson correlation. Two distinct clusters of tumors were observed in the dendrogram shown in figure 4. The
232 first cluster included 9 out of 9 (100%) Met FVPTCs, whereas the second one included 11 out of 13 (84.4%)
233 NonMet FVPTCs. Only two of Met samples were classified erroneously.

234 **miRNA-mRNA interaction analysis of Metastatic and Non-metastatic FVPTCs** The potential
235 interactions between 47 mRNAs and 35 miRNAs differentially expressed in Met vs NonMet lesions were
236 studied using DIANA-miRPath v3.0.

237 According to the above procedure, we identified 34 enriched pathways. Three out of the 34 enriched
238 pathways, the ECM-receptor interaction (hsa04512), the TGF- β signaling (hsa04350) and the Cell Cycle
239 (hsa04110) pathways, were included in the PanCancer Progression Panel used for the mRNAs expression
240 analysis (Table 4).

241 Twenty-nine mRNAs of the ECM-receptor interaction pathway (P-value adjusted = 1×10^{-325}) are targeted
242 by 6 miRNAs (miR-19b-3p, miR-19a-3p, miR-145-5p, miR-361-3p, miR-140-5p and miR-143-3p) that were
243 statistically significant in our analysis. None of these miRNAs targeted mRNAs differentially expressed
244 between Met and Non Met groups.

245 For regarding TGF- β signaling pathway (P-value adjusted = 1.8437×10^{-06}), 54 mRNAs are targeted by 10
246 miRNAs that resulted in statistically significant differences in our analysis. Among these, miR-20a-5p
247 targeted *CUL1*, *SMURF1* and *PPP2R1A*; miR-20b-5p targeted *CUL1* and *PPP2R1A*; miR-15b-5p targeted
248 *PPP2R1A* and *SMURF1*; miR-1276 and miR-140-5p targeted *ZFYVE16*. All of these mRNAs were
249 differentially expressed between Met and NonMet. The Cell Cycle pathway (P-value adjusted = 5.3802×10^{-06}),
250 81 genes are targeted by 11 miRNAs that were statistically significant in our analysis. Among these, 2
251 miRNAs (miR-20a-5p and miR-10b-5p) targeted *CUL1* and 2 miRNAs (miR-15b-5p and miR-30e-5p)
252 targeted *SMC3*. Both of these mRNAs were differentially expressed between Met and NonMet.

253 Discussion

254 DM has a frequency less than 5% of patients with well-differentiated thyroid cancer, however continues to
255 be the most significant clinical feature of thyroid cancer mortality. DM are even more uncommon in
256 FVPTCs, affecting the 1% to 9% of the patients. The rarity of this event and the slow development of this
257 disease determined until now difficulties in collecting a sufficiently large cohort of metastatic FVPTCs.

258 To date, no reliable molecular signature for the metastatic potential in thyroid carcinomas exists. For this
259 reason, to gain insight into the molecular basis underlying the different metastasis ability of FVPTCs with
260 similar characteristics in terms of the histotype, degree of neoplastic invasion and tumor size, we performed
261 a mRNAs and miRNA expression profiling analysis.

262 Given the exploratory nature of the present study to detect differential expressed mRNAs and miRNAs in
263 this kind of lesions, we used an FDR at 0.25. It indicates that the result is likely to be valid 3 out of 4 times.

264 Given the high number of variables and the relatively small cohort of patients recruited, this cutoff represent

265 a good compromise between true-positive and false-positive mRNAs/miRNAs. We compared the mRNAs
266 and miRNA expression profiles between Met and NonMet FVPTCs and discovered 47 mRNAs and 35
267 miRNAs that were differentially expressed between these two groups.

268 Specifically, for the mRNAs expression analysis, we used a panel of 740 mRNAs that provide coverage of
269 several pathways that directly involved in the specific tumor progression processes, such as angiogenesis,
270 extracellular matrix, epithelial to mesenchymal transition and metastasis. It is worth mentioning that some
271 genes participate in these different biological pathways simultaneously.

272 So far, several studies investigated the ability of gene expression profile to explain the biological basis of
273 metastatic processes in various tumor models. Of note, it has been reported that the molecular layout of some
274 genes are tissue-specific; therefore, it has to be contextualized in each specific tumor model (Bock Axelsen
275 et al., 2007). Interestingly, Riker *et al* analyzed the gene expression profile of the primary and metastatic
276 melanomas finding a molecular signature of the few genes involved in the progression or suppression of the
277 metastatic phenotype (Riker et al., 2008). Similarly, Ganepola and collaborators analyzed the gene
278 expression profile of non-metastatic and metastatic colon cancer proving a number of genes that are able to
279 discriminate between the two groups (Ganepola et al., 2010).

280 Despite the recent expansion of knowledge and continuous attempts to characterize the PTCs genetically,
281 and unlike other tumor models, only one study by Londero et al. has been specifically conducted on the
282 metastatic potential of thyroid cancer. In detail, analyzing the gene expression profile in a series of metastatic
283 and non-metastatic PTCs, they identified 17 genes that were differentially expressed between the two groups.
284 However, through validation, no prognostic significance of this classifier was shown (Londero et al., 2016).

285 Our mRNA expression profile analysis allowed the identification of 47 mRNAs that were able to distinguish
286 FVPTCs into two clusters, one including the majority of Met (82%) and the other including the majority of
287 NonMet FVPTCs (75%). In detail, our signature constituted 4 mRNAs that were upregulated in Met
288 FVPTCs compared to NonMet and 44 that were downregulated. In thyroid cancer, no data are available so
289 far regarding the 4 upregulated mRNA; however, these mRNA have been described in the context of other
290 tumor models in which their overexpression seems to be related to cancer progression (Ghiselli and Liu,
291 2005; Chevalier et al., 2016; Ganesan et al., 2008; Zhao et al., 2014; M. Zhang et al., 2013; McSherry et al.,
292 2009; Tian et al., 2015). Our findings showed that all these mRNAs are significantly overexpressed in Met

293 FVPTCs and they could play an important role in thyroid cancer metastasization. The majority of
294 differentially expressed mRNAs evaluated in our study have been found to be downregulated in Met
295 FVPTCs. Our data appear to contrast the findings reported in other studies, in which the largest part of these
296 same genes were found to be upregulated in human non-thyroid carcinomas (such as renal cell carcinoma,
297 breast cancer, gastric cancer, prostate cancer and colon cancer) both in *vivo* and in *vitro* models.
298 Nevertheless, differentially expressed mRNAs related to the different functions (such as extracellular matrix
299 production, cell adhesion, cell migration, growth factor binding and angiogenesis) may be downregulated
300 during malignant transformation in human cell models, as reported by Danielsson and collaborators
301 (Danielsson et al., 2013). Our results are in line with this study demonstrating that the downregulation of
302 some genes could be correlated with metastatic processes. Interestingly, at least some of the 43
303 downregulated mRNAs, (*CUL1*, *TGFRB2*, *NOTCH1*, *NR4A1*, *ADAMTS1*, *SPARC*, *TCF4*) have already
304 been reported in the literature as tumor suppressor genes. Notably, these genes, downregulated in the Met
305 group, are reported to have been downregulated in a variety of tumors including cervical, breast, gastric and
306 colon cancer. Our data, in addition to those previously reported, seem to suggest a key role of these genes in
307 the process of thyroid cancer metastasization (Korzeniewski et al., 2009; Yang et al., 2017; Lobroy et al.,
308 2011; Wu et al., 2017; Martino-Echarri et al., 2015; Chen et al., 2014; Angus-Hill et al., 2011).

309 The complex functions of miRNAs, especially in tumorigenesis, are still poorly understood. However, in the
310 past few years, several studies have evaluated the miRNA profiles of different cancer models, including
311 thyroid tumors (Nikiforova et al., 2009). In these works, different miRNAs have been associated with tumor
312 progression, invasion and metastasis showing that some molecules may also behave as predictive biomarkers
313 in cancer (Zhou et al., 2015). In thyroid cancer, although several works have focused on miRNA expression,
314 no studies investigating a peculiar miRNA profile for metastatic potential exist, particularly in FVPTCs.

315 Overall, in our series both upregulated and downregulated miRNAs suggest that a potential miRNA
316 signature can be useful to distinguish FVPTCs with metastatic potential from FVPTCs that are less likely to
317 produce DM. In the same way, several of these miRNAs (both up and downregulated), have been already
318 described in the context of thyroid cancer and of other tumor models in which their expression seems to be
319 connected to cancer progression (Ma, 2010; Fan et al., 2014; Ahmad et al., 2015; Borrelli et al., 2017; X.
320 Zhang et al., 2013; Xiong et al., 2015; Yuan et al., 2013; Chen et al., 2016; He et al., 2016)

321 In the present study, we also investigated the potential miRNA-mRNA interactions using mRNA and
322 miRNAs that are differentially expressed between the two group of lesions. Seven out of the 35 deregulated
323 miRNAs specifically targeted 5 mRNAs differentially expressed between the two group. In detail, this
324 analysis showed that in the TFG- β signal pathway, 3 upregulated miRNAs in Met lesions (miR-20a-5p, miR-
325 20b-5p and miR-15b-5p) targeted 3 downregulated mRNAs (*CUL1*, *SMURF1* and *PPP2R1A*); 2
326 downregulated (miR-1276 and miR-140-5p) targeted *ZFYVE16* that resulted downregulated in Met group.

327 In the Cell-Cycle pathway, 2 upregulated miRNAs (miR-20a-5p and miR-10b-5p) targeted 1 downregulated
328 mRNA, *CUL1*; whereas *SMC3*, an upregulated mRNA was regulated from 1 downregulated (miR-30e-5p)
329 and 1 upregulated (miR-15b-5p) miRNA. These results seem to be encouraging, however, further
330 examinations are needed to define the role of the miRNA-mRNA interactions in FVPTCs with and without
331 distant metastasis.

332 In conclusion, to the best of our knowledge, this work represents the first attempt to analyze either the
333 mRNA and miRNA expression profiles in primary FVPTCs with and without distant metastases. The main
334 limitation of the present study is represented by the small number of cases, which does not allow us to draw
335 any definitive conclusion about the combination of the mRNA/miRNA molecular profile with other tumor
336 characteristics, such as mutational status and clinicopathological features. Beyond this, we identified a
337 potential molecular signature that consists of 47 mRNAs and 35 miRNAs that can distinguish FVPTC with
338 metastatic potential. Further examinations are needed to validate the possible role of these data in the
339 prognostic risk stratification of the patients.

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346

347 **Disclosure/ Conflict of Interest**

348 The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartially
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- 578

579 **Figure Legends:**

580

581 **Figure 1 Diagram of the study design.**

582 * Samples suitable for the study fulfilling all the inclusion criteria, as specified in Patients and Study Design

583

584 **Figure 2 Representative histopathological images showing similar pathological findings of metastatic**
585 **and non- metastatic follicular variant of papillary thyroid carcinomas (FVPTC).**

586 A and B, Capsular invasion: these encapsulated FVPTCs, metastatic (A) and non-metastatic (B) respectively,
587 are completely surrounded by fibrous capsule with mushroom-shaped images of capsular invasion
588 (hematoxylin and eosin staining [H&E], original magnification X 2.5).

589 C and D, Vascular invasion: in these encapsulated FVPTCs, metastatic (C) and non- metastatic (D)
590 respectively, aggregates of neoplastic cells are seen within the vascular spaces attached to the wall and
591 covered by endothelium (H&E , original magnification X 10).

592 E and F, Nuclear features: these encapsulated FVPTCs, metastatic (E) and non- metastatic (F) respectively,
593 show at high magnification the typical nuclear features of PTC (original magnification X 40).

594

595 **Figure 3 Hierarchical clustering of metastatic and non-metastatic FVPTC using statistically significant**
596 **genes.** The columns represent the samples and the rows represent the genes. Only those genes with a
597 statistically significantly different expression (FDR 0.25) between metastatic and non-metastatic samples
598 were used for the hierarchical clustering. Red and green indicate a high and a low level of expression,
599 respectively. Met, metastatic; NonMet, non-metastatic.

600 **Figure 4 Hierarchical clustering of metastatic and non-metastatic FVPTCs using statistically**
601 **significant miRNAs.** The columns represent the samples and the rows represent the miRNAs. Only those
602 miRNAs with a statistically significantly different expression (FDR 0.25) between metastatic and non-
603 metastatic samples were used for the hierarchical clustering. Red and green indicate a high and a low level of
604 expression, respectively. Met, metastatic; NonMet, non-metastatic.

Table 1 Clinicopathological features of 12 metastatic and 12 non-metastatic Follicular Variant of Papillary Thyroid Carcinomas.

Clinicopathological features	Met (n = 12)	NonMet (n = 12)	P-values
Age at diagnosis (yr), mean \pm SD median; range	58.3 \pm 19.5 63.5; 17-77	46.4 \pm 13.7 50.5; 19-64	0.0621
Male gender (n, %)	3 (25%)	3 (25%)	0.6742
Tumor size (cm), mean \pm SD median; range	3.7 \pm 2.3 3.4; 1.3-9.0	3.2 \pm 1.6 3.0; 0.9-6.0	0.9032
Encapsulated FVPTC (n, %)	11 (91.7%)	10 (83.3%)	0.5032
Infiltrative FVPTC (n, %)	1 (8.3%)	2 (16.7%)	0.5032
Capsular invasion for encapsulated PTC (n, %)	11 (91.7%)	10 (83.3%)	0.5032
Vascular invasion (n, %)	8 (66.7%)	5 (41.7%)	0.2044
Extrathyroidal invasion (n, %)	2 (16.7%)	2 (16.7%)	0.7031
Intra-tumoral fibrosis (n, %)	6 (50.0%)	4 (33.3%)	0.3431
Multifocality (n, %)	5 (41.7%)	6 (50.0%)	0.5043
Bilaterality (n, %)	3 (25.0%)	6 (50.0%)	0.2012
AJCC pT stage (8 th edition)			
- pT1a	1 (8.3%)	1 (8.3%)	0.7612
- pT1b	4 (33.3%)	2 (16.6%)	0.3225
- pT2	4 (33.3%)	5 (41.6%)	0.5056
- pT3a	3 (25.0%)	4 (33.3%)	0.5034
Distant metastasis at presentation	4 (33.3%)	0	0.0433
Follow-up (yr), mean \pm SD median; range	5.1 \pm 3.6 3.5; 1-12	9.3 \pm 2.8 10; 3-9	0.0082
Disease status at the last follow-up			
- dead of unknown cause	3 (25%)	0	0.1053
- dead of disease	2 (16.6%)	0	0.2344
- alive with disease	7 (58.4%)	0	0.0024
- free of disease	0	12 (100%)	<0.0001

FVPTC, Follicular Variant of Papillary Thyroid Carcinoma; Met, metastatic; NonMet, non-metastatic; AJCC, American Joint Committee on Cancer.

Table 2 Differentially Expressed genes in metastatic versus non-metastatic Follicular Variant of Papillary Thyroid Carcinomas.

Upregulated Genes	ANG	EMT	ECM	META	P-value*	P-value adjusted**
<i>SMC3</i>			+		0.0116	0.1996
<i>TOM1L1</i>		+			0.0116	0.1996
<i>PLA2G2A</i>	+				0.0122	0.1996
<i>F11R</i>	+	+	+		0.0193	0.2441
Downregulated Genes	ANG	EMT	ECM	META	P-value*	P-value adjusted**
<i>AP1M2</i>		+			0.0005	0.1537
<i>SMURF1</i>		+	+		0.0015	0.1537
<i>CX3CL1</i>	+				0.0017	0.1537
<i>FERMT2</i>		+			0.0021	0.1537
<i>SRGN</i>		+			0.0031	0.1537
<i>ZFYVE16</i>			+		0.0031	0.1537
<i>LAMA4</i>			+		0.0046	0.1537
<i>RGCC</i>	+				0.0046	0.1537
<i>TAL1</i>	+				0.0046	0.1537
<i>CUL1</i>			+		0.0056	0.1537
<i>EGFL7</i>	+		+		0.0056	0.1537
<i>PNPLA6</i>	+				0.0056	0.1537
<i>ROBO4</i>	+				0.0056	0.1537
<i>TGFR2</i>	+	+	+		0.0056	0.1537
<i>GLYR1</i>		+			0.0067	0.1537
<i>MAP2K2</i>	+			+	0.0067	0.1537
<i>NOTCH1</i>	+	+			0.0067	0.1537
<i>NR4A1</i>	+			+	0.0067	0.1537
<i>SH2B3</i>		+			0.0067	0.1537
<i>ADAMTS1</i>			+		0.0081	0.1537
<i>EPHA1</i>	+	+			0.0081	0.1537
<i>FLT4</i>	+			+	0.0081	0.1537
<i>HSPG2</i>	+		+		0.0081	0.1537
<i>PPP2R1A</i>			+		0.0081	0.1537
<i>SPARC</i>	+	+	+		0.0081	0.1537
<i>TCF4</i>		+			0.0081	0.1537
<i>DLL4</i>	+				0.0097	0.1537
<i>PECAMI</i>			+		0.0097	0.1537
<i>PLCG1</i>	+			+	0.0097	0.1537
<i>PTPRB</i>	+				0.0097	0.1632
<i>SPARCL1</i>		+			0.0097	0.1708
<i>SRPK2</i>	+				0.0097	0.1708
<i>ECSCR</i>	+				0.0111	0.1814
<i>FLT1</i>	+			+	0.0111	0.1944
<i>GIMAP4</i>		+			0.0111	0.1959
<i>ACVRL1</i>	+		+		0.0138	0.2003
<i>MAP3K7</i>	+				0.0138	0.2021
<i>NOS3</i>	+			+	0.0138	0.2082
<i>DICER1</i>	+				0.0138	0.2082
<i>MMRN2</i>	+		+		0.0163	0.2211
<i>TIE1</i>	+				0.0163	0.2211
<i>GDF15</i>		+			0.0192	0.2428
<i>KDR</i>	+				0.0193	0.2441

*P-values were obtained by using Mann-Whitney *U* test

**Adjusted P-values using the Benjamini-Hochberg method

ANG, angiogenesis; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; META, metastasis.

Table 3 Differentially Expressed miRNAs in metastatic versus non-metastatic Follicular Variant of Papillary Thyroid Carcinoma.

Upregulated miRNAs	P-value*	P-value adjusted**
miR-19b-3p	0.0020	0.0708
miR-20a-5p+ miR-20b-5p	0.0071	0.1106
miR-10b-5p	0.0086	0.1106
miR-1972	0.0086	0.1106
miR-125a-5p	0.0126	0.1163
miR-19a-3p	0.0126	0.1150
miR-296-5p	0.0126	0.1150
miR-32-5p	0.0151	0.1154
miR-26b-5p	0.0181	0.1274
miR-30a-5p	0.0181	0.1274
miR-141-3p	0.0215	0.1390
miR-15b-5p	0.0215	0.1390
miR-423-3p	0.0256	0.1484
miR-125a-5p	0.0418	0.2252
miR-135a-5p	0.0488	0.2478
Downregulated miRNAs	P-value*	P-value adjusted**
miR-155-5p	0.0005	0.0708
miR-320e	0.0008	0.0708
miR-1276	0.0019	0.0708
miR-548ah-5p	0.0024	0.0708
miR-130a-3p	0.0039	0.1011
miR-1253	0.0043	0.1011
miR-1973	0.0058	0.1106
miR-361-3p	0.0071	0.1106
miR-140-5p	0.0071	0.1106
miR-1323	0.0080	0.1106
miR-593-3p	0.0102	0.1106
miR-143-3p	0.0104	0.1106
miR-1285-5p	0.0126	0.1150
miR-126-3p	0.0151	0.1154
miR-5196-3p+miR-6732-3p	0.0151	0.1154
miR-630	0.0214	0.1390
miR-145-5p	0.0256	0.1484
miR-4488	0.0256	0.1484
miR-888-5p	0.0302	0.1659
miR-30e-5p	0.0488	0.2478

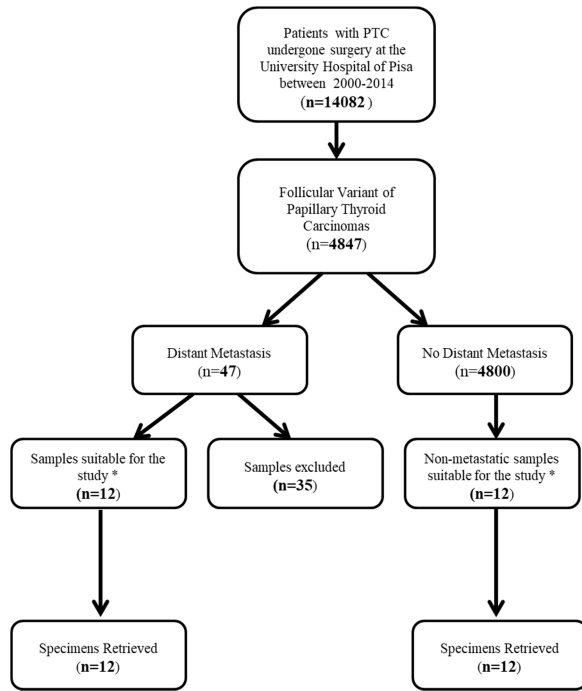
*P-values were obtained by using Mann-Whitney *U* test

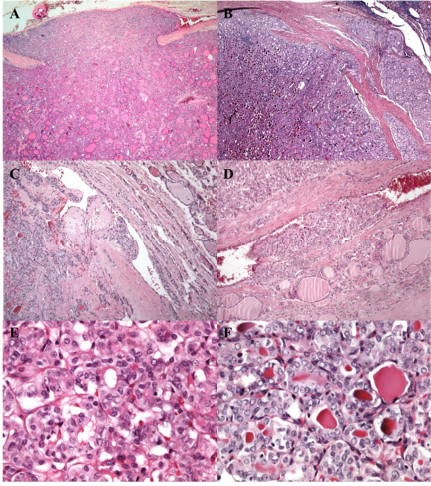
** Adjusted P-values using the Benjamini-Hochberg method

Table 4 Results from the DIANA-miRPath v3.0 predictions of KEGG pathways.

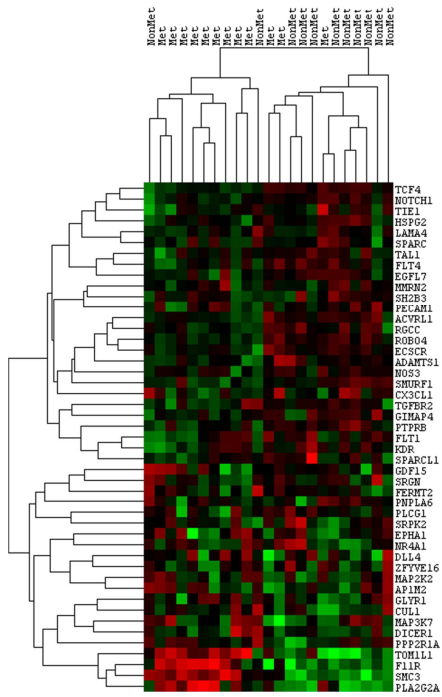
KEGG pathway	P-value adjusted*	#genes	#miRNAs
ECM-receptor interaction	1×10^{-325}	29	6
hsa-miR-19b-3p	0.0005	12	
hsa-miR-19a-3p	0.0021	13	
hsa-miR-145-5p	3.0728×10^{-11}	7	
hsa-miR-361-3p	0.0002	7	
hsa-miR-140-5p	0.0379	5	
hsa-miR-143-3p	3.1179×10^{-30}	16	
TGF-β signaling pathway	1.8437×10^{-06}	54	10
hsa-miR-19b-3p	0.0417	12	
hsa-miR-20a-5p	0.0005	21	
hsa-miR-20b-5p	0.0002	12	
hsa-miR-19a-3p	0.0229	15	
hsa-miR-15b-5p	0.0006	22	
hsa-miR-155-5p	0.0003	11	
hsa-miR-1276	0.0012	5	
hsa-miR-130a-3p	0.0004	19	
hsa-miR-140-5p	0.0162	8	
hsa-miR-145-5p	0.0010	12	
Cell cycle	5.3802×10^{-06}	81	11
hsa-miR-20a-5p	2.0614×10^{-05}	33	
hsa-miR-10b-5p	0.0220	13	
hsa-miR-32-5p	0.0259	19	
hsa-miR-141-3p	0.0126	17	
hsa-miR-15b-5p	0.0076	33	
hsa-miR-135a-5p	0.0125	7	
hsa-miR-1276	0.0154	10	
hsa-miR-130a-3p	0.0148	25	
hsa-miR-140-5p	0.0132	15	
hsa-miR-143-3p	0.0381	13	
hsa-miR-30e-5p	0.0159	24	

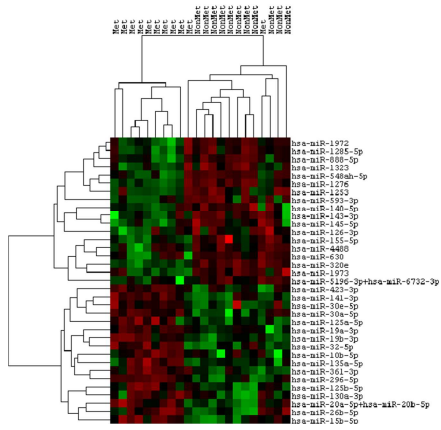
* P-values adjusted using the Benjamini-Hochberg method





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Highlights

- No molecular signatures are currently available for metastatic potential of FVPTCs.
- A set of mRNA and miRNA are significantly deregulated in metastatic tumors.
- A mRNA/miRNA signature could identify FVPTCs at risk to develop metastasis.

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