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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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Abstract

Follicular Variant of Papillary Thyroid Carcinoma (FVPTC) is usually associated with a good outcome.

Nevertheless, in rare cases, it develops distant metastases (1-9%).

Our goal was to investigate whether mRNA and miRNA expression profiles may help distinguish between

metastatic versus non-metastatic FVPTCs.

Twenty-four primary FVPTCs, 12 metastatic and 12 non-metastatic, with similar clinicopathological features

were selected and analyzed by nanoString nCounter technology using two distinct panels for expression

analysis of 740 mRNA and 798 miRNAs. Data analysis was performed using the nanoString nSolver 3.0

software.

Forty-seven mRNA and 35 miRNAs were differentially expressed between the two groups. Using these

mRNA and miRNAs, metastatic and non-metastatic FVPTCs were clearly divided into two distinct clusters.

Our results indicate that FVPTCs with metastatic abilities have different expression profiles compared to the

non-metastatic. A prospective validation is needed to evaluate the usefulness of this molecular approach in

the early identification of high-risk FVPTCs.

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

Introduction

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

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

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

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

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

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

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

45 2016; Xu et al., 2017). In those rare cases, the most common site of metastasis are the lungs followed by the

46 bones. Other metastatic areas are less common and involve the mediastinum, brain, liver and skin (Nwaeze et

47 al., 2015; Daniels, 2016).

48 Higher rates of distant metastases from FVPTCs are described in patients with non-encapsulated forms,

49 extrathyroidal extension, and angiolymphatic invasion (Haq and Harmer, 2005; Li et al., 2016). In particular,

50 some authors reported that 25 to 50% of FVPTCs with vascular invasion show distant metastases (Mete and

51 Asa, 2011).

52 The clinicopathological features of a tumor by themselves are not sufficient to predict the development of a

53 distant metastatic disease in FVPTCs.

54 Important questions about metastatic progression remain to be addressed. Currently, the main challenge is

55 understanding metastatic potential at the molecular level.

56 In this regard, several studies have reported comparisons of the gene expression profiles between primary

57 and metastatic tumors, such as melanoma, breast, colon and lung cancer (Ganepola et al., 2010; Ramaswamy

58 et al., 2003; Weigelt et al., 2003).

59 Furthermore, numerous studies demonstrated that microRNAs (miRNA) play a key role   in metastatic

60 progression. These miRNAs control metastatic potential through the divergent or convergent regulation of

61 metastatic gene pathways (Baranwal and Alahari, 2010; Pencheva and Tavazoie, 2013; Seven et al., 2014).

62 However, unlike other tumor models, very few studies concern the identification of peculiar molecular

63 profiles for the metastatic potential of thyroid cancer exist (Londero et al., 2016). More importantly there are

64 no studies investigating the presence of a molecular signature for the metastatic potential of FVPTCs.

65 Therefore, according to this literature data, we aimed to investigate the molecular basis underlying the

66 different ability of FVPTCs to metastasize with similar histopathological characteristics. In this study, we

67 characterized the molecular landscape of metastatic and non-metastatic FVPTC by analyzing two distinct

68 panels that consist of carefully selected mRNAs and miRNAs.

69 **Materials and Methods**

70 **Patients and Study Design** Between 2000 and 2014, 14,082 consecutive patients underwent thyroid surgery

71 at the Department of Surgical, Medical, Molecular Pathology and Critical Area of the University of Pisa and

4

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 >3/10HPF, 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 $\geq$1.8, and the ratio between the value of absorbance (A) at 260 nm and the one

117 at 230 nm was $\geq$ 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*

6

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

7

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.

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

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

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

Metastatic lesions have been confirmed histologically in 10 out of 12 patients. Nine patients had a single DM located in bone (6), central nervous system (2) and lung (1); one patient developed multiple bone metastases. Two patients had a DM detected in first ^{131}I WBS (multiple bone and thorax uptake).

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

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

Association between Differentially Expressed mRNAs and tumor progression pathways The differentially expressed mRNAs in the Met versus NonMet FVPTCs were implicated in different pathways, as shown in Table 2. In detail, the 4 mRNAs upregulated in the Met group were: *SMC3* (Structural Maintenance Of Chromosomes 3), which is involved in remodeling of basement membrane (ECM process) and tumor growth, *TOM1L1* (target of myb1 (chicken)-like 1), which is implicated in the Epithelial-Mesenchymal Transition (EMT process), *PLA2G2A* (phospholipase A2, group IIA), which is related to VEGFA signaling (ANG) and *F11R* (F11 receptor), which is associated with Blood Coagulation (ANG process), Cell Adhesion and Cellular Differentiation (EMT process), remodeling of the Integral to Membrane 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 $= 1 \times 10^{-325}$) 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 $= 1.8437 \times 10^{-06}$), 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 $= 5.3802 \times 10^{-06}$)

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

11

265 a good compromise between true-positive and false-pasitive 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 us 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)

13

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.

14

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Disclosure/ Conflict of Interest

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

of the research reported.

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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 ± SD	58.3 ± 19.5	46.4 ± 13.7	0.0621
median; range	63.5; 17-77	50.5; 19-64	
Male gender (n, %)	3 (25%)	3 (25%)	0.6742
Tumor size (cm), mean ± SD	3.7 ± 2.3	3.2 ± 1.6	0.9032
median; range	3.4; 1.3-9.0	3.0; 0.9-6.0	
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 (8th 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 ± SD	5.1 ± 3.6	9.3 ± 2.8	0.0082
median; range	3.5; 1-12	10; 3-9	
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**
SMC3			+		0.0116	0.1996
TOM1L1		+			0.0116	0.1996
PLA2G2A	+				0.0122	0.1996
F11R	+	+	+		0.0193	0.2441

Downregulated Genes	ANG	EMT	ECM	META	P-value*	P-value adjusted**
AP1M2		+			0.0005	0.1537
SMURF1		+	+		0.0015	0.1537
CX3CL1	+				0.0017	0.1537
FERMT2		+			0.0021	0.1537
SRGN		+			0.0031	0.1537
ZFYVE16			+		0.0031	0.1537
LAMA4			+		0.0046	0.1537
RGCC	+				0.0046	0.1537
TAL1	+				0.0046	0.1537
CUL1			+		0.0056	0.1537
EGFL7	+		+		0.0056	0.1537
PNPLA6	+				0.0056	0.1537
ROBO4	+				0.0056	0.1537
TGFR2	+	+	+		0.0056	0.1537
GLYR1		+			0.0067	0.1537
MAP2K2	+			+	0.0067	0.1537
NOTCH1	+	+			0.0067	0.1537
NR4A1	+			+	0.0067	0.1537
SH2B3		+			0.0067	0.1537
ADAMTS1			+		0.0081	0.1537
EPHA1	+	+			0.0081	0.1537
FLT4	+			+	0.0081	0.1537
HSPG2	+		+		0.0081	0.1537
PPP2R1A			+		0.0081	0.1537
SPARC	+	+	+		0.0081	0.1537
TCF4		+			0.0081	0.1537
DLL4	+				0.0097	0.1537
PECAM1			+		0.0097	0.1537
PLCG1	+			+	0.0097	0.1537
PTPRB	+				0.0097	0.1632
SPARCL1		+			0.0097	0.1708
SRPK2	+				0.0097	0.1708
ECSCR	+				0.0111	0.1814
FLT1	+			+	0.0111	0.1944
GIMAP4		+			0.0111	0.1959
ACVRL1	+		+		0.0138	0.2003
MAP3K7	+				0.0138	0.2021
NOS3	+			+	0.0138	0.2082
DICER1	+				0.0138	0.2082
MMRN2	+		+		0.0163	0.2211
TIE1	+				0.0163	0.2211
GDF15		+			0.0192	0.2428
KDR	+				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, ephitelial-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

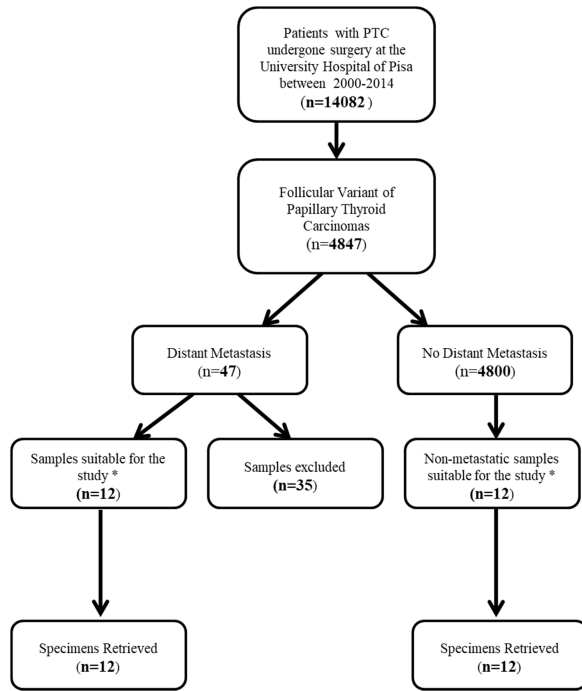| Downregulated miRNAs	P-value*	P-value adjusted**
miR-155-5p	0.0005	0.0708
miR-320e	0.0008	0.0708
miR-1276	0.0019	0.0708
miR-548ah-5p	0.0024	0.0708
miR-130a-3p	0.0039	0.1011
miR-1253	0.0043	0.1011
miR-1973	0.0058	0.1106
miR-361-3p	0.0071	0.1106
miR-140-5p	0.0071	0.1106
miR-1323	0.0080	0.1106
miR-593-3p	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

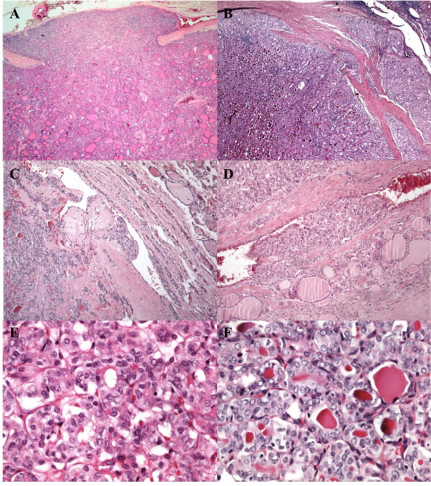*P-values were obtained by using Mann-Whitney *U* test

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

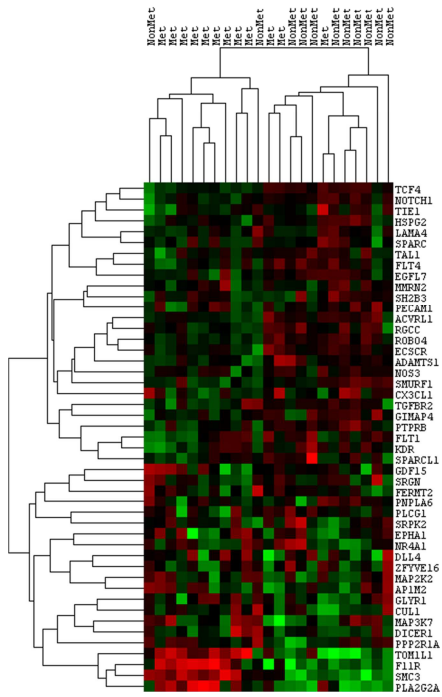**Table 4** Results from the DIANA-miRPath v3.0 predictions of KEGG pathways.

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

Patients with PTC
undergone surgery at the
University Hospital of Pisa
between 2000-2014
(**n=14082**)

Follicular Variant of
Papillary Thyroid
Carcinomas
(n=**4847**)

Distant Metastasis
(n=**47**)

No Distant Metastasis
(n=**4800**)

Samples suitable for the
study *
(**n=12**)

Samples excluded
(**n=35**)

Non-metastatic samples
suitable for the study *
(**n=12**)

Specimens Retrieved
(**n=12**)

Specimens Retrieved
(**n=12**)
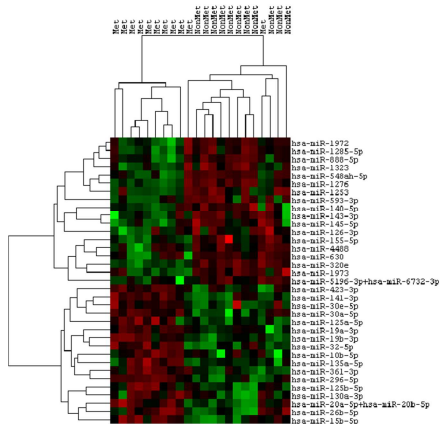
Highlights

- 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.