Identification of *SPRY4* as a novel candidate susceptibility gene for familial non-medullary thyroid cancer[1]

Inês J. Marques[1,2,3], *Inês Gomes[1] , *Marta Pojo[1] , Carolina Pires[1] , Margarida M. Moura[1] , Rafael Cabrera[4] , Catarina Santos[5] , Wilfred F.J. van IJcken[6] , Manuel R. Teixeira[5,7], José S. Ramalho[2,3], Valeriano Leite[3,8], Branca M. Cavaco[1]

[1]Unidade de Investigação em Patobiologia Molecular (UIPM), Instituto Português de Oncologia de Lisboa Francisco Gentil, Lisboa, Portugal; [2]Chronic Diseases Research Centre (CEDOC), Universidade Nova de Lisboa, Lisboa, Portugal; [3]Nova Medical School/Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal; [4]Serviço de Anatomia Patológica, Instituto Português de Oncologia de Lisboa Francisco Gentil, Lisboa, Portugal; [5]Serviço de Genética, Instituto Português de Oncologia do Porto Francisco Gentil, Porto, Portugal; [6]Center for Biomics, Erasmus University Medical Center, Rotterdam, The Netherlands; [7]Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal; [8]Serviço de Endocrinologia, Instituto Português de Oncologia de Lisboa Francisco Gentil, Lisboa, Portugal

1 INTRODUCTION

Thyroid cancer is the most common malignancy of the endocrine system. The great majority derive from the thyroid follicular cells and are designated as Non-Medullary Thyroid Carcinomas (NMTC). NMTC may present as a familial form, being designated as **Familial Non-Medullary Thyroid Carcinoma (FNMTC)**[2].

FNMTC is defined by the diagnosis of two or more first degree relatives with thyroid cancer, and family members frequently present benign lesions of the thyroid (*e.g.* multinodular goiter - MNG)[2]. Although some susceptibility genes for FNMTC have already been identified (*e.g. NKX2-1*[3], *FOXE1*[4], *DICER1*[5], *CHEK2*[6]), these are mutated only in a small number of families. Therefore, the genetic basis of FNMTC has remained largely unknown, representing a limitation for molecular diagnosis and clinical management.

IPO-Lisboa is a reference centre for the treatment of this malignancy and our group has collected one of the biggest cohorts of FNMTC families worldwide.

Aim: To identify new susceptibility genes for FNMTC through whole-exome sequencing (WES) analysis of leukocyte DNAs of patients from a highly informative FNMTC family.

2 MATERIALS AND METHODS

• Whole-exome sequencing (WES)

Genomic DNA samples obtained from peripheral blood leukocytes from six representative members of one family affected with thyroid cancer were used for WES (Figure 1) using Agilent SureSelect Human All Exon enrichment. Bioinformatic analyses were undertaken in order to filter and select the genetic variants shared by the affected members, which were subsequently validated by Sanger sequencing. To select the most likely pathogenic variants, several studies were performed, including family segregation analysis, *in silico* impact characterization, and gene expression (mRNA and protein) depiction in databases. As a result of these analyses, a germline variant in the *SPRY4* gene (c.701C>T; p.Thr234Met) was found to be the most promising candidate genetic variant to proceed for functional characterization.

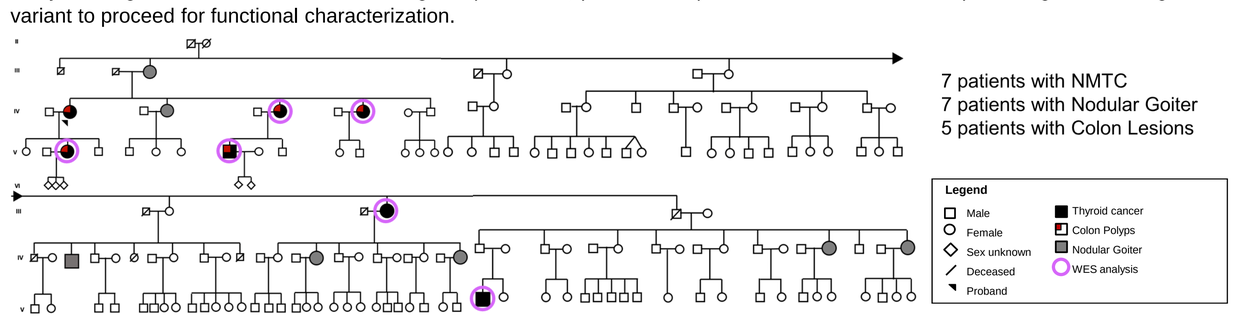


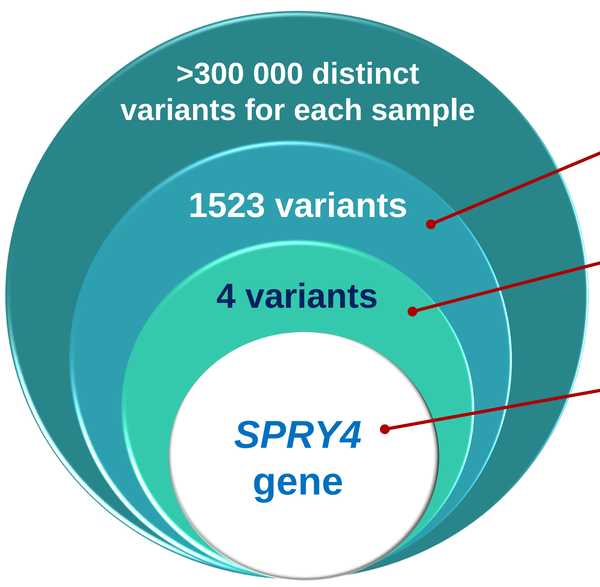
Figure 1. Pedigree of the FNMTC family analyzed in this study

• *In vitro* studies

To investigate the biological consequences of *SPRY4* p.Thr234Met germline variant in cell transformation, functional assays were performed to assess clonogenicity, cell survival, cell cycle and migration, using PCCL3, TPC-1 and NIH/3T3 cells, expressing the wild-type (WT) and mutant (MUT; c.701C>T, p.Thr234Met) *SPRY4* sequences. The phosphorylation profiles of cancer-related kinases in TPC-1 cells expressing WT and MUT *SPRY4* was also explored, using a human phospho-kinase antibody array. Western blot was performed for validation. Additionally, the effect of Trametinib, a highly specific and potent MEK1/2 inhibitor, was evaluated in WT and MUT *SPRY4* TPC-1 cells viability.

3 RESULTS

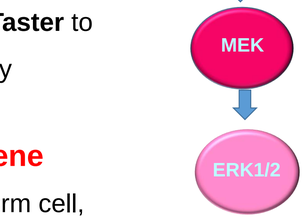
Selection of a candidate susceptibility gene through WES analysis



- European population frequency < 1%
- Validation through Sanger sequencing
- Family segregation analysis
- Pathogenic potential (protein impact, signalling pathways, gene expression)
- Absence in 150 Portuguese healthy controls
- Gene function

SPRY4 Gene (sprouty RTK signaling antagonist 4)

- SPRY4 protein has a **signal transducer activity** and is **ubiquitously expressed** in embryonic and adult tissues, **including the thyroid**
- Inhibits ERK activation through binding to Raf1 in a Ras-independent way
  → **MAPK/ERK signaling pathway**
- Modulates proliferation, differentiation, motility and cell survival
- The variant c.701C>T was predicted by **SIFT**, **PolyPhen**, and **MutationTaster** to be **deleterious**, **probably damaging**, and **disease causing**, respectively



Tumour supressor
Medullary thyroid cancer, prostate, breast, colon cancers, melanoma

Oncogene
Testicular germ cell, ovarian cancers

Functional characterization of SPRY4 c.701C>T (p.Thr234Met) variant

- Mutant (MUT; c.701C>T, p.Thr234Met) *SPRY4* **increased colony formation**

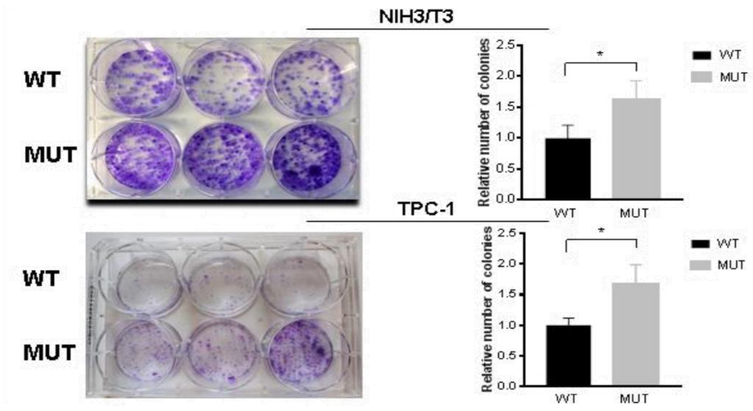


Figure 2. Representative 6-well plate from a colony formation assay using NIH/3T3 and TPC-1 cells.

- MUT*SPRY4* **increased viability** in all cell models

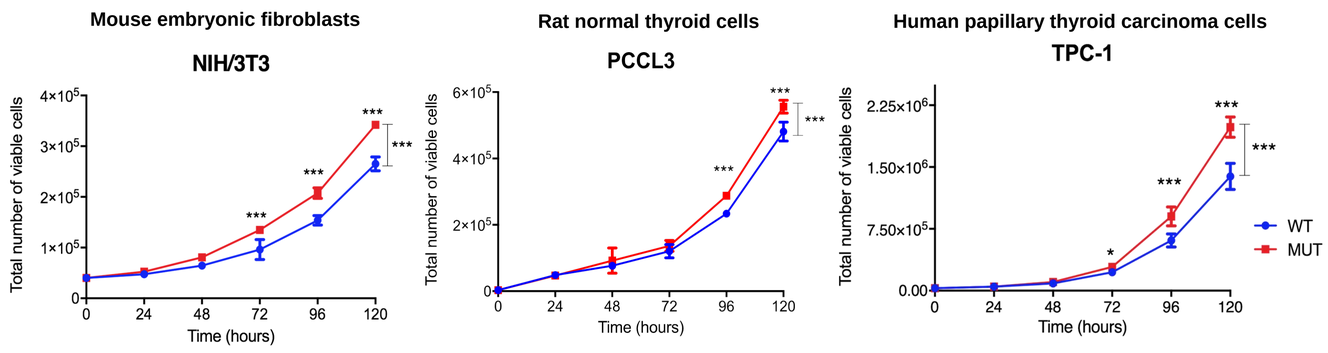


Figure 3. Cell viability over time in cells expressing wild-type (WT) and mutant (MUT) *SPRY4*, by direct cell counting at different time-points.

siRNA-mediated *SPRY4* gene silencing:
Decrease of TPC-1 viable cells, suggesting that this gene may have an **oncogenic activity** in follicular cell derived thyroid cancer

Human phospho-kinase antibody array:
MUT *SPRY4* **increased phosphorylation of MAPK/ERK targets** (*e.g.* STAT3, STAT5b, MSK1/2, and p53)

- Western blot analysis showed an **increase in ERK phosphorylation levels** in TPC-1 MUT, suggesting that an increase in ERK activity could be associated with SPRY4 mutated status

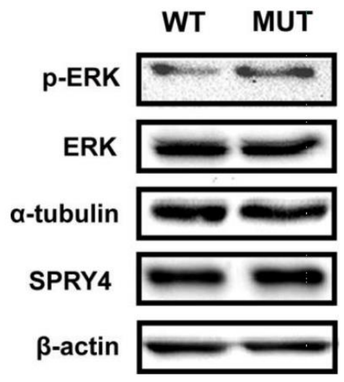


Figure 4. Effect of WT and MUT SPRY4 in ERK kinase activity in TPC-1 cells.

- Trametinib (MEK inhibitor) treatment **decreased cell viability**, effect that was **more pronounced in MUT cells**, supporting that the proliferative advantage may result from addiction to MAPK/ERK signaling

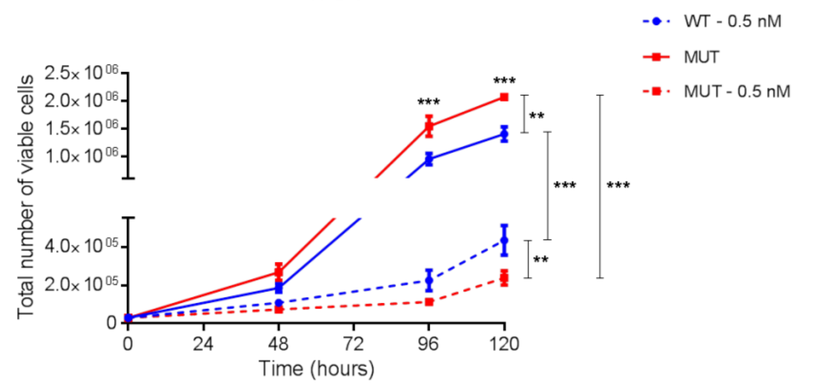


Figure 5. Cell viability over time of TPC-1 cells expressing WT and MUT *SPRY4*, with and without treatment with trametinib.

4 CONCLUSIONS

- WES analysis and functional assays in one family identified *SPRY4* as a likely novel candidate susceptibility gene for FNMTC, allowing a better understanding of the cellular and molecular mechanisms underlying thyroid cancer development.
- The effects of the *SPRY4* variant seem to be mediated through the MAPK/ERK pathway. However, further studies may provide insights into its function and whether it is a susceptibility gene for other cases of FNMTC.

REFERENCES

[1] Marques, IJ *et al.* (2021) *Thyroid*, (ja);
[2] Pinto, AE *et al.* (2014) *Eur J Endocrinol*, **170**:321-327;
[3] Ngan, ESW *et al*. (2009) *JNCI J Natl Cancer Inst*, **101**:162–175
[4] Pereira, JS *et al.* (2015) *Endocrine*, **49**:204–214;
[5]. Rio Frio, T *et al.* (2011) *JAMA*, **305**:68–77;
[6] Zhao, Y *et al.* (2020) *Thyroid*, **30**:924-930.

ACKNOWLEDGMENTS

Thyroid®