Assessment of *SPAG9* Transcript in Fine Needle Aspirates of Thyroid Nodules

Bertrand Volard^b Sophie Krieger^{a, b} Gaétane Planchard^b Agnès Hardouin^b
Dominique Vaur^b Jean-Pierre Rame^c Stéphane Bardet^d

^aEA 1772, University of Basse-Normandie, and Departments of ^bBiopathology, ^cHead and Neck Surgery, and

^dNuclear Medicine and Thyroid Unit, Centre François Baclesse, Caen, France

Key Words

Sperm-associated antigen 9 • Fine needle aspiration •
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Abstract

Objectives: Sperm-associated antigen 9 (*SPAG9*) has been suggested as a possible biomarker in several malignancies including thyroid cancer. We investigated the expression of *SPAG9* mRNA in fine needle aspiration (FNA) material from papillary thyroid carcinoma (PTC) and benign thyroid nodules. **Study Design:** *SPAG9* expression was assessed in 36 FNA samples corresponding to 16 PTC and 20 benign nodules using the original method detecting the *SPAG9* transcript containing intron 21 (NCBI X91879). The presence of the *BRAF* V600E point mutation was also analyzed by pyrosequencing. **Results:** Six of 16 (38%) PTC samples were positive for X91879 *SPAG9* transcript compared to 8 of 20 (40%) benign samples ($p = 0.88$). Out of 12 *BRAF*-positive PTC, 3 (25%) also expressed the *SPAG9* transcript compared to 3 out of 4 *BRAF*-negative PTC (75%; $p = 0.12$). **Conclusions:** The X91879 *SPAG9* transcript originally described does not appear to be overexpressed in FNA material from PTC or to be clinically relevant in the diagnosis of thyroid nodules.

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Introduction

Fine needle aspiration (FNA) cytology of thyroid nodules is considered as the key tool to distinguish between benign and malignant tumors [1]. However, FNA cytology is classified as indeterminate in approximately 20–30% of patients who exhibit a variable risk of cancer estimated at 5–15 and 60–75% depending on the cytological Bethesda subgroup [2]. Surgery is generally advocated in this category of patients for obtaining pathological proof, even though it can be considered unnecessary a posteriori in a substantial proportion of patients with benign nodules. Recently, molecular analysis for a panel of mutations (*BRAF*, *RAS*, *RET/PTC*, *PAX8/PPAR γ*) has shown promising diagnostic value especially in patients with indeterminate cytology [3, 4]. The sensitivity of this molecular approach is still limited, however, and further molecular markers are probably needed to improve its usefulness in routine [5].

In more than 70% of papillary thyroid carcinomas (PTC), activating events that involve one gene of the mitogen-activated protein kinase (MAPK) signaling pathway are detected, such as the V600E mutation of the *BRAF* gene or *RET/PTC* rearrangement [6]. Interestingly, the structural organization of MAPK appears to be facilitated by scaffold proteins such as members of the

c-Jun-NH2 terminal kinase-interacting protein (JIP) family [7]. In this context, sperm-associated antigen 9 (SPAG9), a single-copy gene mapped to human chromosome 17q21 [8] and recently a new member of the JIP family [9], was found to be expressed either as a protein or a transcript in 78% of selectively chosen thyroid carcinoma specimens and not in benign nodules [10]. This gene has been involved in sperm-egg fusion, and its expression was found to be a biomarker in several cancers [11–16]. To our knowledge, SPAG9 expression has never been investigated in FNA material of thyroid nodules. The aim of this study was to examine the feasibility and the diagnostic relevance of assessing the expression of SPAG9 transcript in FNA samples from pathologically proven malignant and benign thyroid nodules.

Materials and Methods

Patients and Samples

The present work was carried out as part of a prospective study conducted at our center in 2010–2011 to assess the diagnostic value of a panel of molecular markers including BRAF V600E in patients with clinical thyroid nodules. FNA of the palpable nodule was performed by the surgeon, immediately before thyroidectomy, in an anesthetized patient. All samples were obtained with informed consent from each patient. SPAG9 mRNA analyses were performed in the first 20 benign samples and 16 malignant samples of that cohort. The 20 benign nodules included 7 follicular adenomas, 11 hyperplastic nodules, 1 pseudo-nodule with Hashimoto's thyroiditis and 1 Hürthle cell adenoma. The malignant tumors corresponded to 16 PTC including 9 with the common form and 7 with the follicular variant. According to the 2009 TNM classification (7th edition), PTC were pT1bNxM0 (n = 2), pT1bN0M0 (n = 4), pT2NxM0 (n = 3), pT2N0M0 (n = 2), pT3N0M0 (n = 2), pT3N0M1 (n = 1), pT4aN0M0 (n = 1) and pT4aN1aM0 (n = 1). Regarding FNA methods, three needle passes were obtained in each patient. The two first passes were dedicated for cytology. The third one, devoted for molecular biology, was collected in 400 µl of RNA later® (QIAGEN, Valencia, Calif., USA), a RNA stabilization reagent, and frozen at –20°C until analysis.

Nucleic Acids Extraction

Total RNA and DNA were extracted from FNA samples using AllPrep DNA/RNA Micro Kit® (QIAGEN) according to the manufacturer's protocol. The amount of total RNA and DNA was determined by spectrophotometry using NanoVue® (GE Health Care Bio-Science, Piscataway, N.J., USA) and used as template for RT-PCR and PCR amplification.

SPAG9 Transcript Detection

SPAG9 transcript expression was analyzed using the method originally described by Garg et al. [11] detecting the X91879 (NCBI) SPAG9 transcript including intron 21. The X91879 SPAG9 transcript was amplified using a One-Step RT-PCR Kit® (Qiagen) according to the manufacturer's protocol, with the same forward

primer 5'-GACAGAGATGATTTCGGGCATCACGAGAAAA-3', and reverse primer 5'-CTAAGTTGATGACCCATTATTATACCTCGACTG-3', used by Garg et al. [11]. The expected RT-PCR products length was 1,245 bp. β-Actin mRNA expression was used as internal control. The RT-PCR products were separated by electrophoresis on a 2% agarose gel in the presence of ethidium bromide and photographed under UV light. All RT-PCR products were sequenced using BigDye Terminator Kit® on an ABI 3730® (Applied Biosystems, Foster City, Calif., USA) to confirm primer specificity. Each RT-PCR was performed in duplicate.

BRAF Point Mutation Analysis

The presence of the BRAF V600E point mutation was analyzed by pyrosequencing. PCR and sequencing primers were designed using PSQ Assay Design Software® (Qiagen): F-Biot: 5'-CTTC-ATAATGCTTGCTCTGATAGG-3', R: 5'-GGCCAAAATT-AATCAGTGGAA-3', sequencing primer R 5'-CCACTCCATC-GAGATT-3'. PCR amplification was performed on 10 ng of total DNA using the Pyromark PCR Kit® (Qiagen) according to the manufacturer's protocol. PCR-products were used as template for pyrosequencing using Pyromark Gold Q24 Reagents Kit® (Qiagen) on the Pyromark Q24® (Qiagen). DNA obtained from patients with wild-type and BRAF V600E mutated colorectal cancer were used as negative and positive controls, respectively. These internal controls were pyrosequenced with PCR products.

Statistical Analysis

Data were compared using the χ^2 test or the Fisher exact test when appropriate. p values below 0.05 were considered statistically significant.

Results

Using the original gel electrophoresis method, X91879 SPAG9 transcript expression was found in FNA samples from 6/16 (38%) PTC compared to 8/20 (40%) benign nodules (p = 0.88; fig. 1). X91879 SPAG9 transcript was positive in 6 PTC including 3 common forms and 3 follicular variants, in 3 follicular adenomas and 5 hyperplastic nodules. Of the 16 PTC, 12 (75%) were positive for BRAF V600E mutation including tumors classified pT1bNxM0 (n = 1), pT1bN0M0 (n = 4), pT2NxM0 (n = 1), pT2N0M0 (n = 1), pT3N0M0 (n = 2), pT3N0M1 (n = 1), pT4aN0M0 (n = 1) or pT4aN1aM0 (n = 1). Three of these 12 BRAF-positive PTC (25%) also expressed the SPAG9 transcript compared to 3 out of 4 BRAF-negative PTC (75%; p = 0.12).

Discussion

In the present study, we analyzed the expression of the X91879 SPAG9 transcript in FNA material of benign thyroid nodules and PTC using the method originally de-

Fig. 1. Expression of X91879 (NCBI) *SPAG9* transcript in FNA samples from benign nodules (**a**) and PTC (**b**). The specificity of each *SPAG9* band was confirmed by the Sanger sequencing method on an ABI 3730. β -Actin expression was used as an internal control. B = RT-PCR blank, negative control; MWM = molecular weight marker. **a** Results in FNA samples from benign nodules

($n = 20$). HN = Hyperplastic nodule; HCA = Hürthle cell adenoma; FA = follicular adenoma; HT = Hashimoto's thyroiditis. Note that a thin band was distinguishable in lanes 5 and 13. **b** Results in FNA samples from PTC ($n = 16$). CF = Common form; FV = follicular variant. The *BRAF* V600E mutational status is reported for each sample.

scribed by Garg et al. [11]. In contrast to the data by Garg et al. [10], we found that the X91879 *SPAG9* transcript was not specific of malignant tumor FNA samples, but was equally expressed in approximately 38–40% of both benign nodules and PTC samples. Similar discrepancies have been reported in breast cancer. Although *SPAG9* mRNA was detected in up to 90% of early-stage breast cancers and 97% of low-grade tumors in one study [14], such a high expression of *SPAG9* was not found in breast cancer cell lines in another study [17].

There are no clear explanations for the discrepancies between the present data and those of Garg et al. [10]. Differences might be related to the fact that we tested FNA samples, and not tumor tissues. The findings of similar results in tumor tissues would have strengthened this negative study. However, no frozen tumors but only paraffin-embedded specimens were available for this study. On the latter, testing *SPAG9* expression was not possible, due to the limited quality of the extracted nucleic acids. Nevertheless, the finding that 75% of PTC were positive for *BRAF* V600E mutation validates that FNA samples did correspond to tumor tissues. The high proportion of

BRAF mutations for our geographic area [18] may be partly related to the number of high risk T3–T4 tumors (approximately 30% in our series) and to the use of a highly sensitive pyrosequencing technique.

In conclusion, the X91879 *SPAG9* transcript originally described does not appear to be overexpressed in FNA material of PTC or to be clinically relevant in the diagnosis of thyroid nodules.

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Disclosure Statement

We declare having no conflict of interest.

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