IMPACT OF PREGNANCY ON PROGNOSIS OF DIFFERENTIATED THYROID CANCER: CLINICAL AND MOLECULAR FEATURES.

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ABSTRACT

Objective: Differentiated Thyroid Cancer (DTC) commonly occurs in women of child-bearing age and represents the second most frequent tumor diagnosed during pregnancy only behind breast cancer. It is possible that associated physiological changes could favour tumor development and growth. However, few data are available about the outcome of DTC related to pregnancy, leading to conflicting results. **Methods**: 340 patients with DTC <45 years old were retrospectively studied. Patients were divided into three groups according to the time of tumor diagnosis respect of pregnancy. Group 1: diagnosis of DTC at least 2 years after delivery, Group 2: diagnosis during pregnancy or within the second year after delivery, Group 3: nulliparous patients at the time of diagnosis. We evaluated clinical outcome and immunohistochemical expression of estrogen receptor α (ERα), estrogen receptor β (ERβ), progesterone receptor (PGR) and aromatase. We also analysed the gene expression of NIS and the prevalence of BRAF V600E mutations. **Results**: Persistence/recurrence of disease was significantly higher in group 2 patients than control groups (p: 0.023). No significant differences were observed in other clinical parameters. Furthermore no difference among the groups were recorded about ER pattern, NIS expression and BRAF mutations. **Conclusions**: Persistence/recurrence of DTC is significantly higher in pregnant patients, suggesting that pregnancy could really exert a negative prognostic role in patients with DTC. The underlying mechanisms are not yet clarified and further studies are required. Our results suggest that a more careful follow up is needed when diagnosis of DTC occurs during pregnancy or shortly after.

Abbreviations

DTC: differentiated thyroid cancer; rhTSH: Recombinant human thyroid stimulating hormone; Tg: tireoglobuline; S-Tg: tireoglobuline measured under suppressive therapy with l-T4; A-Tg: tireoglobuline measured at ablation with I-131; rhTSH-Tg: tireoglobuline measured after stimulation with rhTSH; AbTg: Anti-Tireoglobuline Antibody; MTS: metastasis; ER: estrogen receptor; AR: androgen receptor; PGR: progesterone receptor; RAI; radioiodine ablations with I-131; NIS: Na-I symporter

INTRODUCTION

Differentiated Thyroid Cancer (DTC) is a relatively rare neoplasia. It represents 3.6% of all malignant tumors in the United States (SEER Cancer Statistics Review, National Cancer Institute Surveillance, Epidemiology, and End Results; 1975–2005. Available from http://seer.cancer.gov) and it is generally characterized by good prognosis. Consequently, studies evaluating the prognosis of this tumor have to consider a wide number of cases and a long-term follow-up to highlight differences in survival or disease recurrence rate. The majority of relapses usually occurs within 5 years from the initial treatment, and only sporadic cases have been subsequently documented (1, 2). Despite the low incidence, in the USA DTC represents the second most frequent diagnosed tumor during pregnancy, only after breast cancer. In women of child-bearing age about 10% of thyroid carcinomas is diagnosed during pregnancy or early after delivery (SEER Cancer Statistics Review, National Cancer Institute Surveillance, Epidemiology, and End Results; 1975–2005. Available from http://seer.cancer.gov). These findings have led to hypothesize that during this period the presence of several physiologic changes, such as hormonal secretion, growth factors and negative iodine balance, could create a favourable environment for the development and growth of tumors.

However, only few studies about the outcome of DTC related to pregnancy have been published. A recent review (3) reports that pregnancy is not generally described in literature as a determining condition for prognosis of DTC, neither in terms of DTC-related death (4), nor of overall survival (5, 6) .

Nevertheless, these findings are in contrast with the more recent study published by Vannucchi et al. (7), who reported that DTC in pregnant women had a significant increase of persistent/recurrent disease than those in non pregnant patients. Since the parameters and the methodology in each study were very different, their results were not easily comparable. In fact, the studies conducted by Yasmeen et al. (5) and Herzon et al. (6), focused mainly on the overall survival, while the study of Moosa and Mazzaferri (4) had DTC-related death and disease recurrence, evaluated by biopsy or by 131-I uptake in distant sites, as primary focus. On the contrary, Vannucchi et al. (7) have evaluated persistent/recurrent DTC through more sensitive tests, such as basal and stimulated Tg levels after exogenous TSH injection (rhTSH, Thyrogen®, Genzyme Corporation, Sanofi Company, Cambridge, MA) which have not been used in the other studies and may - at least partially - explain the different conclusions.

Moreover, Vannucchi et al. (7) observed a significantly higher immunohistochemical expression of the Estrogen Receptor α (ERα) in tumors from pregnant women compared to the control groups. With special reference to hormone receptor expression in thyroid tumors, a recent study (8) on the immunoistochemical expression of estrogen and androgen receptors (AR) in DTC showed that ERα was acquired or increased in tumor samples as compared to the corresponding normal tissue, whereas AR and ERß expression was decreased in tumors compared with the surrounding normal tissue. These patterns appeared also to be associated with the clinical behaviour, being the high

expression of ERα and AR and the low expression of Erß associated with a more aggressive phenotype.

In such a controversial situation, we therefore designed the present study to characterize at clinical, phenotypical and molecular levels DTC cases in pregnancy as compared to matched control groups.

MATHERIAL AND METHODS

Patients

We retrospectively evaluated more than 1200 medical records of patients with DTC treated and followed up from 2001 to 2011 at the Nuclear Medicine Department of Mauriziano Hospital, which covers up to 80% of all radioiodine ablations with I-131 (RAI) performed in the Piedmont region. This allowed us to obtain an extremely homogeneous population, representative of DTC epidemiology in the fertile women population.

Among them, 340 women were selected according to the following inclusion criteria:

- Age ≤ 45 years at the time of surgery
- Total thyroidectomy
- I-131 radioiodine ablation
- L-T4 TSH-suppressive therapy (TSH ≤0.1 mU/l) (9)
- Follow up ≥ 1 year
- rhTSH test during follow up or persistent disease (S-Tg > 2 ng/ml)

Patients were divided into three groups according to the time of diagnosis of DTC respect to pregnancy. Group 1 included women (n=152, median age 40, range 25-45) with DTC

diagnosis at least 2 years after delivery. Group 2 included women (n=38, median age 35, range 26-41) with diagnosis of DTC during pregnancy or within two years after delivery. Group 3 included nulliparous patients at the time of diagnosis (n=150, median age 30, range 15-45).

Tumors were classified following the World Health Organization classification (10), staged according to the 6th edition of TNM staging (American Joint Committee on Cancer, AJCC). (11) and classified as Low and High Risk according to the European Consensus Statement criteria (9). ETA guidelines divide patients into three groups: Very Low, Low and High Risk but the first group was not represented in our series because it includes patients with no indications for RAI .

Remission or persistent/recurrent disease were defined according to the European and American guidelines for the management of DTC (9, 12):

- REMISSION: S-Tg and rh-TSH-Tg < 0,6 mcg/l, negative AbTg and normal neck ultrasound.
- PERSISTENT/RECURRENT disease at least one of the following criteria:
  - S-Tg > 2 µg/L
  - rh-TSH-Tg > 2 µg/L
  - Persistence of AbTg > 4 years with a trend to increase (an increasing antibody production or newly antibodies appearance as a consequence of an increased of autoantigen production) (13, 14)
  - Neck or distant MTS
  - Radioiodine uptake outside thyroid bed

Serum Tg levels were measured during L-T4 withdrawal, immediately before RAI and after 12 months of L-T4 suppressive therapy. Then, patients received one injection of rh-TSH (0.9 mg i.m., Thyrogen®, Genzyme Corporation, Sanofi Company, Cambridge, MA) for two consecutive days; serum samples for TSH and Tg measurements were collected on days 0 (before first rh-TSH administration), 3 and 4. Neck ultrasonography was performed 6 and 12 months after RAI. TSH levels were evaluated using a chemiluminescent immunoassay (Access Immunoassay Systems, Beckman Coulter®, Inc.), Tg levels were determined using chemiluminescent immunoassay (Access Immunoassay Systems, Beckman Coulter®), with a functional sensitivity of 0.6 µg/L; Ab-Tg antibodies were detected with chemiluminescent immunoassay (Access Immunoassay Systems, Beckman Coulter®, Inc.). Based on Tg assay, we considered 0.6 µg/L as the cut-off value between undetectable and measurable Tg levels, according to Mazzaferri *et al.* (1)

Immunohistochemical analysis of hormone receptors and aromatase in tumor tissues

Immunohistochemical evaluation of estrogen receptor α (ERα), estrogen receptor ß (ERß), progesterone receptor (PGR) and aromatase were performed in 37 histological specimens selected from the three different groups (12 of group 1, 10 of group 2, 15 of group 3). Cases for immunohistochemical analysis were blinded selected to obtain 3 groups homogenous in terms of age and stage, regardless of the outcome.

Immunohistochemical analyses were carried out on paraffin-embedded tissue sections of 5 µm, after dewaxing, dehydration in alcohol and rehydration in PBS pH 7,5. Endogenous peroxidase block was performed by immersion of the slides in 0,3% solution of methanol and hydrogen peroxide for 15 minutes. Then sections were incubated with the following monoclonal primary antibodies: ERα (clone 1D5, dilution 1:300, Dako, Glostrup, DK), ERβ

(clone PPG5/10, dilution 1:50, Dako), PGR (clone 636, dilution 1:300, Dako), and Aromatase (clone mca2077s, dilution 1:50, Serotec, Kidlington, UK). A biotin-free, dextran chain-based detection system (EnVysion, Dako) and diaminobenzidine as the chromogen were used according to standard protocols. All markers were assessed in tumoral and peritumoral tissue using H-score evaluation, which takes into account both quantitative and qualitative expression with a 0-300 range scale.

Molecular analysis

Nucleic acids isolation. Genomic DNA was isolated from formalin-fixed, paraffin-embedded tissues using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). RNA was isolated from paraffin embedded material using the high pure RNA paraffin kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The quantity of isolated DNA and RNA was assessed using a Biophotometer (Eppendorf, Hamburg, Germany).

BRAF point mutation analysis. The presence of *BRAF* point mutation (V600E) was analysed using pyrosequencing and PCR primers following previously published protocols (15). PCR amplification for the pyrosequencing assay was performed according to standard protocols. The amplicons were mixed with sequencing primers and sequencing was performed using a PyroGold reagent kit (Biotage AB) according to the manufacturer's protocol. Results were analyzed using the PSQ-96 MA 2.0.2 software (Biotage).

Quantitative real time PCR for the sodium/iodide symporter.

Relative cDNA quantitation of the sodium/iodide symporter (NIS) and an internal reference gene (β-actin) were done using a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (Taqman); Applied Biosystems/Life technologies, Foster City, CA]. Beta-actin primers and probe were previously published (16), whereas for NIS the TaqMan gene expression assay 20X (SLC5A5

Hs00166567_m1, Applied Biosystems) was used according to manufacturer's intructions. The PCR reaction mixture consisted 1,200 nmol/L of each primer, 200 nmol/L probe, 200 nmol/L each of dATP, dCTP, dGTP, dTTP, 3.5 mmol/L MgCl2, and 1x Taqman Universal PCR Master mix to a final volume of 20 µL (all reagents were from PE Applied Biosystems,). Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. To analyze target gene expression in individual tumors, the relative gene expression levels were expressed as ratios (differences between the C_t values) between 2 absolute measurements (genes of interest/internal reference gene). Then, the $\Delta\Delta C_t$ values were calculated subtracting ΔC_t values of each case to the value of the normal sample expression, and converting the ratio by the $\mathbf{2^{-\Delta\Delta C_t}}$ formula; cases were considered of low or high expression according to the median expression level obtained.

Statistical analysis

The clinical (age, outcome, number of treatments, ablation-Tg levels, High/Low risk classification) and pathological/molecular features (histology, pTNM stage, hormone receptor expression, NIS gene expression and BRAF mutation status) were compared among the three groups of patients by using the χ^2 test for dichotomic variables and the Mann Whitney and Kruskal-Wallis tests for continuous variables, as appropriate. The reciprocal correlation among immunohistochemical markers was evaluated using the Spearman's test. Statistical significance was defined as p<0,05.

A logistic multivariable analysis was performed. Dependent dichotomous variable was tumor persistence/recurrence (1) or remission (0). Age, T, N and multifocality of primary tumor and pregnancy (DTC diagnosis during pregnancy or within 2 years after delivery: 1;

Other groups: 0) were the independent variables. All these analyses were performed using STATISTICA for Windows Ver. 8.0.

RESULTS

Clinical, biochemical, histopathological and molecular parameters in the three groups are reported in Table 1.

No significant differences were noticed in the number of treatments for achieving clinical remission, in the tumor size or extrathyroidal invasion, in the lymphnodal metastatic involvement at diagnosis, in histology and in High Risk/Low Risk classification of patients according to the ETA guidelines (9).

Clinical remission was obtained in 150/152 patients (98.7%) of group 1, 34/38 patients (89.5%) of group 2 and in 143/150 patients (95.3%) of group 3. Persistent/recurrent disease was observed in 2/152 patients (1.3%) of group 1, 4/38 patients (10.5%) of group 2 and in 7/150 patients (4.7%) of group 3. Our results showed a significant difference (χ^2 : 7.532, P= 0.023) in the outcome among the three groups, with a greater percentage of persistent disease in group 2 than in group 1 and 3. Group 1 and 3 did not show any significant difference. Only 4/38 patients in group 2 had cytological diagnosis while pregnant. They underwent thyroidectomy in the early postpartum period, achieving clinical remission, showing that the surgical delay of few months was not a factor that could influence the worst outcome of group 2.

As regards the expression of hormone receptors (Figure 1), the percentage of intratumoral and peritumoral expression of ERα in the 37 histological samples were globally low, with no detection of significant differences between the groups (P=0.96). ERβ showed a high expression in the peritumoral tissue in a large number of cases, while in tumoral tissue its

expression was quite variable, similarly in the three groups (P=0.82). PGR expression was mostly negative in peritumoral tissue, while it was quite variable in tumoral tissue, in a similar way in the three groups (P=0.41). A significant correlation was observed in tumor tissue between ERα and PGR (Spearman's R value: R=0.49; P=0.002). Aromatase was negative both on peritumoral and tumoral tissue in all the samples analyzed. BRAF V600E mutation, known as a negative prognostic factor (18), was detected in 25% in group 1, 44,4% in group 2 and 60% in group 3 (average of whole samples= 43%). The difference was not statistically significant (P=0.191) showing that the worst outcome observed in patients of group 2 is independent from BRAF mutation. However, BRAF was mutated in 100% of patients with persistence of disease and in 37,5% of patients in remission, irrespective of the group.

NIS gene expression levels were also not different in the three groups (P=0.82) nor associated to BRAF mutation status (P=0.55).

Logistic multivariable analysis performed on the whole population (thus excluding molecular analyses) showed pregnancy (Group 2) as the unique independent variable for persistent/recurrent DTC prediction. The relative risk (RR) was 1.12 (95% CI 1.02-1.22; p=0.02). Age, T, N and multifocality of the primary tumor did not enter the model.

In the 37 patients with molecular and immunohistochemical data available, BRAF mutation and low NIS expression were strong independent predictors of persistence/recurrence of DTC (Table 2), whereas ERα and PGR did not enter the model. Pregnancy and ERβ positivity were of borderline statistical significance. .

Power analysis was performed grouping the entire population into patients with DTC during or within 2 years after delivery (38 subjects) vs all other patients (302 subjects), with values of 79% and 87%,. by two-sided and one-sided tests, respectively.

DISCUSSION

Thyroid cancer discovered during pregnancy represents a challenge for the clinicians because, at present, there are still no reliable data available supporting a specific management of pregnancy-associated DTCs. Currently pregnant patients with a citologically suspicious thyroid nodule for DTC do not require surgery during pregnancy except in cases of rapid nodular growth and/or the appearance of lymph node metastases (19).

Most studies showed that pregnancy did not worsen the prognosis of DTC. In four studies, the prognosis of women with DTC diagnosed either during pregnancy or within the first postpartum period was compared to that of women diagnosed at another time as controls. In three of these works (4-6), no difference was found in DTC prognosis between pregnant women and control groups. However, in the fourth study (7), Vannucchi et al. reported a significant worse outcome in pregnant patients. As a matter of fact, they observed 60% of recurrent/persistent disease in pregnant women (group 2) *vs* 4.2% in women with DTC diagnosed more than 1 year after delivery (group 1) and 13.1% in nulliparous patients (group 3). Moreover, a higher expression of ERα in tumor samples of pregnant women was reported.

In order to verify these conflicting results, we selected a homogeneous population, dividing patients into three groups according to the criteria adopted by Vannucchi et al.. We extended group 2 to women with DTC diagnosis within 2 years after delivery instead of 1 year, arbitrarily assuming that in tumors with low biological aggressiveness, such as DTC, pregnancy-induced hyperestrogenism may exert its tissue activity in a longer period. At our knowledge no published data are available on this issue. Moreover, in our population the rate of persistent/recurrent disease in patients diagnosed within 1 year or between 1 and 2 years after delivery was very similar (9,5% - 2/21 cases - and 11,7% - 2/17 cases – respectively). On the contrary, all the patients (14/14) diagnosed between 2 and 3 years

after delivery displayed clinical remission.

Consistent with the data reported by Vannucchi et al, we confirmed a significant correlation between pregnancy and a worse outcome of DTC (p= 0,023), representing the unique independent variable for persistent/recurrent disease prediction.

Indeed, thyroid cancer diagnosed during pregnancy (group 2) was found to be significantly associated with persistence or relapse of DTC compared to those diagnosed more than 2 years after delivery (group 1) or before pregnancy (group 3).

Taken together, recent evidence supports the hypothesis that pregnancy may negatively affects the prognosis of DTC. The discrepancy with previous studies could be attributed to the different criteria used for the outcome evaluation, as suggested elsewhere (3). Previous papers used the overall survival, DTC-related death and disease recurrence (evaluated by biopsy or whole body scan) as outcome criteria, which were probably not appropriate for a long survival disease with frequent indolent course. In the present study, according to Vannucchi et al., the persistence/recurrence of disease was investigated using more sensitive and precocious markers such as basal and rhTSH stimulated thyroglobulin and neck ultrasonography, as suggested by European and American guidelines (9, 12).

Nevertheless, the worst outcome in patients of group 2 cannot be referred to a higher prevalence of a worse staging at the time of diagnosis or to a more aggressive histological phenotype because, in our study, no significant differences in the examined clinical and morphological parameters were observed.

The mechanisms by which pregnancy could affect the DTC outcome are not easily explainable. In order to verify whether molecular and/or phenotypical features were influencing the results above, we tested the protein expression of sex hormone receptors, as well as the gene expression of NIS and the prevalence of BRAF mutations in the three groups. Indeed, we cannot support the negative prognostic role of estrogens, as previously

suggested (7), considering that our results did not show any significant expression of ERα and no differences among the three groups were observed. The discrepancy between these results has to be clarified, but a difference in the methodological approach could be considered. For example different antibody dilutions were used in the two works (1:300 vs 1:100 dilution). However, it has to be noted that the good correlation between the low expression of ERα and PGR justifies the reliability of our findings. The immunohistochemical analysis was performed also for the detection of ERβ, showing a variable expression without any significant difference among the three groups of patients. Furthermore, aromatase expression resulted generally very low, leading us to keep out its potential pathophysiological role.

In the multivariable logistic regression analysis, $BRAF^{V600E}$ mutations are associated with a worse prognosis, but their similar distribution among the groups excludes a pathophysiological role on the poorer outcome of group 2 patients .

We hypothesized that the worse outcome of group 2 could be explained by a lower response to radioiodine therapy. As resulted by the multivariable logistic regression analysis, NIS lower expression is associated with a higher persistence/recurrence of DTC, but its distribution was not different among the three groups, excluding a role in affecting the outcome of group 2.

In conclusion, our results, obtained in a large homogeneous population, confirm that pregnancy could really exert a negative prognostic role, at least in terms of risk of persistent disease or recurrence, in patients with differentiated thyroid cancer. Further studies are needed to clarify the pathophysiological mechanisms. At the present state of our knowledge, a more careful follow up is needed when diagnosis of DTC occurs during pregnancy or shortly after. However, the impact on DTC prognosis is not so heavy to justify the reconsideration of the American guidelines for the management of thyroid cancer during pregnancy (19).

DECLARATION OF INTERESTS

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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AUTHOR CONTRIBUTIONS

Ilaria Messuti and Stefania Corvisieri equally contributed to the drafting of this work.

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FIGURE LEGENDS

Figure 1. Immunohistochemical analysis of hormone receptors. A case of multifocal papillary carcinoma (group 3) (**a;** H&E, original magnification 40x), with the predominant nodule of the follicular variant (**b;** H&E, original magnification 200x), and high expression

of ERα (**c**), ERβ (**d**) and PGR (**e**) (c-d-e: immunoperoxidase, original magnification 200x); **f:** scatter plot graphs of the distribution of hormone receptors in the three groups of DTC.

TABLES

Table 1. Clinical, histological and molecular characteristics of patients with a DTC diagnosis at least 2 years after delivery (Group 1), during pregnancy or within two years after delivery (Group 2), or before pregnancy/nulliparous (Group 3)

* Group 2 was significantly different as compared to both group 1 and group 3.

** The ablation-HTG cut-off was defined according to Webb et al. (17)

Table 2: Logistic regression analysis for persistence/recurrence of DTC

Table 1

	Group 1	**Group 2**	**Group 3**	***P* value**
Age at diagnosis (years): Median (range)	40 (25-45)	35 (26-41)	30 (15-45)	<0.001
Duration of follow up (years): Median (range)	5 (1-27)	6 (1-10)	6 (1-20)	0.31
Remission	150/152 (98.7%)	34/38 (89.5%)	143/150(95.3%)	0,023*
Persistence/recurrence	2/152 (1.3%)	4/38 (10.5%)	7/15 (4.7%)	
Number of treatments	1,19	1,21	1,28	0,22

(average)				
Ablation-HTG <10 ng/ml **	127/152 (83,5%)	27/38 (71%)	110/150 (73,3%)	0,060
Ablation-HTG >10 ng/ml **	25/152 (16,5%)	11/38 (29%)	40/150 (26,7)	
High Risk	68/152 (44.7%)	19/38 (50%)	79/150 (52,7%)	0,38
Low Risk	84/152 (55.3%)	19/38 (50%)	71/150 (47,3%)	
TNM (T<3)	105/152 (69%)	26/38 (68.4%)	95/150 (63.3%)	0,85
TNM (T>3)	47/152 (31%)	12/38 (31.6%)	55/150 (36.7%)	
TNM (N -)	114/152 (75%)	29/38 (76.3%)	102/150 (68%)	0,54
TNM (N +)	38/152 (25%)	9/38 (23.6%)	48/150 (32%)	
Histology (High Risk)	54/152 (35.5%)	16/38 (42.1%)	62/150 (41.3%)	0,85
Histology (Low Risk)	98/152 (64.5%)	22/38 (57.9%)	88/150 (58.7%)	
ERα tumor expression	3/12 (25 %)	3/10 (30 %)	4/14 (28.6 %)	0.96
ERβ tumor expression	5/12 (41.7%)	5/10 (50%)	7/15 (46.7%)	0.92
PGR tumor expression	4/12 (33.3%)	3/10 (30%)	8/15 (53.3%)	0.419
BRAF V600E mutation	3/12 (25%)	4/9 (44.4%)	9/15 (60%)	0,19
NIS fold change <1	8/12 (66,6%)	5/8 (62,5%)	9/13 (69,2%)	0,9

Table 2

Variable	**RR (95% CI)**	***P value***
Pregnancy	1.26 (0.97-1.55)	0.09
Erβ positive staining	0.69 (0.38-1.00)	0.06

Presence of BRAF mutation	1.46 (1.16-1.77)	0.005
High NIS expression	0.66 (0.36-0.96)	0.03

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