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Blockade of the programmed death ligand1 (PD-L1) as potential therapy for anaplastic thyroid cancer

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Abstract

Purpose: Anaplastic thyroid carcinoma (ATC) is a rare, highly aggressive form of thyroid cancer (TC) characterized by an aggressive behavior and poor prognosis, resulting in patients' death within a year. Standard treatments, such as chemo and radiotherapy, as well as tyrosine kinase inhibitors, are ineffective for ATC treatment. Cancer immunotherapy is one of the most promising research area in oncology. The PD-1/PD-L1 axis is of particular interest, in light of promising data showing a restoration of host immunity against tumours, with the prospect of long-lasting remissions.

Methods: In this study, we evaluated PD-L1 expression in a large series of TCs (20 cases) showing a progressive dedifferentiation of the thyroid tumour from well differentiated TC to ATC, employing two different antibodies [R&D Systems and VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody]. We also tested the anti PD-L1 mAb in an *in vivo* animal model.

Results: We found that approximately 70-90% of ATC cases were positive for PD-L1 whereas normal thyroid and differentiated TC were negative. Moreover, all analyzed cases presented immunopositive staining in the endothelium of vessels within or in close proximity to the tumour, while normal thyroid vessels were negative. PD-L1 mAb was also effective in inhibiting ATC growth in an *in vivo* model.

Conclusions: These data suggest that immunotherapy may be a promising treatment specific for ATC suggesting the need to start with clinical TRIALS.

Keywords: PD-L1, PD-1, Anaplastic thyroid cancer, immunotherapy

Introduction

Cancer immunotherapy is one of the most promising research area in oncology (1). One approach is the blockade of immune check-points (ICPs), a series of inhibitory pathways crucial for maintaining self tolerance which are deregulated in cancer (1). Because ICPs are initiated by ligand-receptor interactions, they can be blocked by antibodies. Such mechanisms represent an opportunity to enhance antitumor immunity. In particular, the blockade of the programmed cell death protein 1 (PD-1) has been investigated with promising results in several cancers (2-4). PD-1 (B7-1) is a cell-surface glycoprotein normally expressed by macrophage lineage cells and T cells. The binding of PD-1 to one of its ligands, PD-L1 or PD-L2, can inhibit cytotoxic T-cell immune responses, leading to tolerance of cells expressing PD-L1 or PD-L2 (5). PD-L1 is constitutively expressed by tumor cells as a result of oncogenic signalling or dynamic IFN γ expression in tumor microenvironments (6).

Thyroid cancer (TC) is mostly curable with conventional therapies including surgery, hormonal therapy and radioiodine providing clinical remission in nearly 90% of cases. However, a small group of TCs is more aggressive, developing distant metastases and being refractory to conventional therapies (7). This group includes about 10% of well differentiated TCs, most of the poorly differentiated TCs and the anaplastic TC (ATC). In particular, ATC represents 1–2% of thyroid malignancies and is believed to arise from terminal dedifferentiation of papillary or follicular TC (8). ATC is characterized by high mitotic rate, lymphovascular invasion and undifferentiated features which confer on ATC an extremely aggressive behaviour and a very poor prognosis, resulting in patients' death within a year (9). Tyrosine kinase inhibitors are ineffective in the treatment of ATC (9-11).

A few studies have addressed the expression of PD-L1 in TC both in papillary thyroid cancer (PTC) and poorly differentiated/ATC (12-18). Interestingly, finding a correlation between PD-L1 expression and the aggressive behavior of thyroid disease, they concluded that identification of PD-L1 expression may be exploited to uncover a new therapeutic strategy for patients with refractory TC.

In this study we evaluated PD-L1 expression by immunohistochemistry in a large series of TCs, from the poorly differentiated forms of cancer to the ATC, employing two different antibodies [VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody and the anti-PD-L1 mAb of the R&D Systems]. We also tested the efficacy of anti PD-L1 antibody as tools for TC immunotherapy in an *in vivo* animal model.

28 **Materials and Methods**

29 **Patients and tissues**

30 The study group comprises 20 patients with ATC from the Histopathological sections of the University Hospital of
31 Siena and from Arezzo Hospital, where the histopathological diagnoses were performed. Two cases (10%) were
32 biopsies from a neck inoperable mass, while 18 (90%) were surgical resections of thyroid tumours. All samples but
33 three (85%) also included normal thyroid gland, which was useful to check PD-L1 immunoreactivity in normal thyroid
34 tissue, while a progressive dedifferentiation of the thyroid tumour was seen in 13 samples (65% of cases). This striking
35 peculiarity of the present files often allowed us to observe different aspects of the tumours in the same section, i.e.
36 different histological grades as well as different degrees of immunoreactivity. Among these 13 samples, 12 (87.5%)
37 dedifferentiated from the insular variant of papillary TC to ATC, 1 (12.5%) evolved directly from papillary TC to ATC.
38 According to the World Health Organization (WHO) classification of tumours, carcinomas were classified as well-
39 differentiated (PTC), poorly differentiated (insular variant of PTC) and anaplastic (19). A synopsis of patient's
40 characteristics is reported in table 1. Supplementary figure 1 shows representative images of the files studied.

41 The histopathological diagnosis was based on the histomorphological aspect of the tumor and on clinical data. Every
42 case showed a high proliferative index (Ki67, 40-70%), positivity for TP53, negativity for TTF-1; other tumours that
43 could display a similar morphology were excluded by performing immunohistochemical markers such as CD34, CD31,
44 desmin, smooth-muscle actin, S-100 protein, HMB45, Melan A, CD45. Such analyses were performed at the time of the
45 original pathological report and therefore different clones and products were used.

46 Informed consent to use biological specimens for investigational procedures was obtained from all patients or their
47 relatives and the study was conducted in accordance with our local ethical committee.

48

49 **Immunohistochemistry**

50 All samples were fixed in buffered formalin for 12-48 hours. After fixation, the surgical samples were examined,
51 sampled for pathological diagnosis, and paraffin-embedded. Significant selected block(s) were used for the study.
52 Criteria used for block selection were tumor areas with scarce or absent necrotic phenomena and, when possible,
53 transition areas including within single blocks normal thyroid tissue, well differentiated thyroid tumor, poorly
54 differentiated thyroid tumor and ATC. In most cases, to document the progression of the neoplastic disease two
55 different slides were required and used. Immunohistochemistry was carried out employing two different antibodies and
56 two different revealing methods.

57 In one protocol, deparaffinized sections underwent antigen retrieval with citrate buffer (pH 6.0) for 10 minutes at 98°. 58 After rinsing in PBS, unspecific protein-protein interactions were quenched with 5% BSA in PBS for 10 minutes. Then, 59 sections were incubated with anti-PD-L1 mAb (CAT #MAB1561, R&D Systems, Minneapolis, MN) for 60 minutes, 60 followed by ImmPRESS AP reagent kit anti-mouse IgG for 30 minutes according to manufacturer instructions (Vector, 61 Burlingame, CA). Upon washings, alkaline phosphatase reaction was developed for 30 minutes with Vector Red 62 alkaline phosphatase substrate kit (Vector, Burlingame, CA) additioned with 2 mM levamisole. Finally, sections were 63 counterstained with hematoxylin. Negative controls were carried out replacing the primary antibody with PBS. 64 In the second protocol, immunohistochemistry was carried out with BenchMark IHC/ISH instruments using 65 VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody which is intended for laboratory use in the detection 66 of the PD-L1 protein in formalin-fixed, paraffin-embedded tissue. Tissues were stained with OptiView DAB IHC 67 Detection Kit on a VENTANA BenchMark Series automated staining instrument. To evaluate and better locate the 68 reaction in neoplastic cells consecutive sections were always stained with H&E. 69 Any staining in necrotic areas was disregarded. Normal human term placenta tissue was used as a positive control for 70 PD-L1 staining. We considered a sample positive for PD-L1 expression only if $\geq 25\%$ of the cells were positively 71 stained. When only scattered neoplastic cells were focally positively immunostained (about 10%), the case was 72 classified as negatively stained, yet we mentioned such a positivity. 73 Evaluation of the immunohistochemical reactions was independently performed by two expert pathologists (PT, RO). 74 Results were compared.

75

76 **Cell culture**

77 Human 8505c ATC cells were obtained from Sigma-Aldrich and maintained in high glucose DMEM supplemented with 78 10% FCS. 8505c cells are known to carry the V600E BRAF mutation.

79

80 **Animal model**

81 Studies were conducted in compliance with institutional guidelines and regulations and after approval (#776/2017-PR). 82 Twelve 5-weeks-old female BALB/c nude mice were purchased from Envigo and maintained in our animal facility in 83 pathogen-free condition. Six animals were inoculated with 6×10^6 ATC cells subcutaneously and treated with PD-L1 84 mAb (Ventana) i.p. at 0.1 mg/dose/mouse on days 1, 3, 5 and 7 after the appearance of a palpable mass. To assess 85 tumor growth, tumor diameters were measured with a calliper and tumor volume calculated using the formula $\text{width}^2 \times$ 86 $\text{length}/0.52$. The remaining six animals, seemingly inoculated with ATC cells, were left untreated (without

87 administration of anti-PD-L1 antibody) and used as a control group. Since data were not normally distributed, Mann-
88 Whitney test was used for statistical analysis. $p < 0.05$ was considered significant.

89

90

Results

PD-L1 expression in ATC: comparison between two different antibodies

ATC cells showed an intense and diffuse plasma membrane and/or cytoplasm positivity (figure 1A) in 13/20 (65%) cases analyzed with VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. In all immunoreactive cases, the reaction was strong and consistent, being positively stained at least 80% of undifferentiated cells. Seven out of 20 (35%) ATC cases were negative (figure 1B), even though in 1 case an intense positive reaction could be observed in isolated scattered cells (about 10% of neoplastic cells). Well-differentiated thyroid cancer of the papillary histotype (13 cases) was always negative as shown in figure 1C in which we could observe the transition from the negative PTC to the positive ATC. Ten poorly differentiated insular tumours were completely negative, while two showed isolated scattered positive cells (Supplementary figure 2). Normal thyroid tissue was analyzed in 17 cases. All of them were negative for PD-L1 expression (figure 1D). Similarly, figure 1E showed a case of ATC with cords of neoplastic cells, positively stained, embedded in a fibrous stroma which was negative for PD-L1 expression (case 10, table 1). Positive control carried out on placenta sections showed a strong reaction on villous epithelium (figure 1F).

When the anti-PD-L1 mAb from R&D systems was tested, we observed that 4 out of 17 (23.5%) normal thyroid cases analyzed displayed a positive staining for PD-L1 and 18/20 (90%) ATC samples had a diffuse and strong membrane positivity (figure 2A). Again, PTCs were negative whereas the infiltrating ATCs were positive for PD-L1 (figure 2B). Insular variant showed focal PD-L1 positive staining in plasma membrane, restricted to the basal part of the cell in 36% of the cases. All analyzed cases presented immunopositive staining in the endothelium of vessels within or in close proximity to the tumor, while normal thyroid vessels were negative. Figure 2C shows insular thyroid cells (on the left) and normal thyroid (on the right) both negative for PD-L1 expression while endothelial cells are positive.

For both antibodies used, we considered a sample positive if $\geq 25\%$ of cells were PD-L1 immunoreactive.

Treatment with PD-L1 mAb reduces tumor growth in *in vivo* model

To evaluate the potential effect of PD-1-PD-L1 blockade *in vivo*, athymic nu/nu mice were injected with the anaplastic 8505c cells. After 20 days, mice were sacrificed and the tumor explanted to verify PD-L1 expression by immunohistochemistry. Figures 3A and 3B show a strong positive reaction in tumours from two different mice employing the VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody.

As 8505c tumours strongly expressed PD-L1, we repeated the experiment and, after the appearance of a palpable mass (day 7 after inoculation), we started with i.p. injection of PD-L1 mAb (0.1 mg/dose/mouse). As shown in figure 3C,

121 mAb was administered on days 1, 3, 5 and 7 after the appearance of tumor masses corresponding to days 7, 10, 12 and
122 14 after inoculation. Tumor volume was calculated using the formula $\text{width}^2 \times \text{length} / 0.52$ and expresses as mm^3 .
123 Treatment with anti-PD-L1 mAb significantly ($p < 0.05$) reduced tumor growth starting from day 17 and this difference
124 lasted until day 23 when mice were sacrificed (figure 3).

125

126 **Discussion**

127 ATC represents 1–2% of the thyroid malignancies and is believed to arise from terminal dedifferentiation of follicular
128 or PTC. ATC shows an aggressive behavior and usually death occurs within 1 year from diagnosis. Actually, a
129 treatment for ATC is lacking and neither tyrosine kinase inhibitors nor standard chemo/radiotherapy are really effective.
130 The blockade of PD-1 has been investigated with promising results in several cancers (20) including few studies on
131 ATC (12-18).

132 This report provides further evidence for the usefulness of anti-PD-L1 mAb treatment against ATC and non
133 differentiated thyroid cancer. A peculiarity of our study is that we compared the efficiency of two different antibodies,
134 the VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody and the anti-PD-L1 mAb of the R&D Systems.
135 We observed that approximately 70% of the ATC cases were positive with the Ventana system and we reached 90% of
136 positive staining with R&D Systems antibody. The latter antibody was apparently more sensitive even in detecting the
137 target in normal thyroid tissue (23.5% of positive sample compared to 0% revealed with Ventana), a finding that has
138 been recently reported also by other investigators (21). The issue of antibody sensitivity in detecting PD-L1 is important
139 and to a certain extent controversial. Indeed the border between higher sensitivity and lower specificity can be subtle
140 and difficult to set. In our case, the higher sensitivity of the R&D Systems antibody could be also due to a certain
141 degree of non-specific staining. Additional comparative tests are needed to confirm the reliability of the R&D Systems
142 antibody. For the same reason, though in a different context, comparative studies of immunohistochemistry assays have
143 been devised with four among the most frequently employed antibodies to detect PD-L1 (22, 23). The monoclonal
144 antibodies tested for this purpose were clones 22C3, 28-8, SP263, SP142, and 73-10. Whereas the former three
145 antibodies showed a comparable sensitivity, clone SP142 was less efficient to stain PD-L1-positive cells and clone 73-
146 10 resulted more sensitive. One of the antibodies tested in the comparative study, clone SP263, was also employed in
147 our research.

148 Another characteristic of our series is the selection of cases in which a progressive dedifferentiation of the tumor from
149 PTC (or the insular variant of PTC) to ATC was present. We noted that PTC or poorly differentiate TC are often
150 negative and become positive when tumour dedifferentiates to ATC. By the use of the anti-PD-L1 mAb from R&D
151 system, endothelial cells of vessels in close proximity to or within the tumor appear positively stained. PD-L1 is
152 expressed in basal condition by human and murine blood endothelial cells (24, 25). Such expression is enhanced by
153 IFN γ and further increases with TNF α (25). Expression of PD-L1 on tumor-associated CD31-positive cells and on
154 tumor-associated lymph vessels has been previously reported (26, 27). This finding is intriguing as it unveils an
155 additional possible target for anti-PD-L1 immunotherapy. Actually, the combination of anti-PD-L1 and anti-VEGFR2

156 was a successful therapy in mice bearing some types of cancer. Apparently, in addition to contrasting the inhibitory
157 loop PD-L1/PD1, the double treatment promoted the formation of high endothelial venules and enhanced tumor
158 lymphocyte infiltration (28).

159 To investigate the role of PD-L1 mAb *in vivo*, we inoculated BALB/c nude mice with human ATC cells. The genetic
160 background of these mice allows the development of graft tumors. Due to the *nu* allele on chromosome 11 they have a
161 dysfunctional rudimentary thymus. They display a much reduced lymphocyte population composed almost entirely of
162 B-cells and a relatively normal IgM response to thymus-dependent antigens. They also have T-cell precursors in their
163 bone marrow. After injection of human ATC cells, tumors were allowed to grow for 20 days and then analyzed for PD-
164 L1 expression. We demonstrated that human ATC cells implanted in mice are positive for PD-L1. Because of the lack
165 of T cells, this expression is probably due to the presence of PD-L1 on B cells, endothelial tumor vessels and dendritic
166 cells. Then, blocking for three weeks PD-1/PD-L1 interaction *in vivo* using a PD-L1 mAb, we could document a
167 significant reduction in tumor volume in anti-PD-L1 treated animals compared to controls, suggesting that
168 immunotherapy may be a promising treatment specific for ATC. Alternatively, this response could be also linked to an
169 antibody-dependent cell cytotoxic mechanism taking advantage of NK or macrophages released by the hematopoietic
170 organs of nude mice. In order to clarify this issue, the data gathered *in vivo* need to be corroborated by other
171 independent experiments. At any rate, our data support the idea that PD-L1 can be a good target for immunotherapy.

172 Actually, there are 4 ongoing clinical trials (ClinicalTrials.gov) addressing the use of immunotherapy agents in thyroid
173 cancer. Two of them (#NCT03072160, # NCT02628067), employ the antibody Pembrolizumab. In particular, study
174 NCT02628067 is recruiting patients with multiple types of advanced (unresectable and/or metastatic) solid tumours that
175 have progressed on standard of care therapy including thyroid cancer (histology not specified). Study-NCT03072160,
176 on the other hand, is intended for patients who have recurrent or metastatic medullary thyroid cancer and surgery is no
177 longer a curative option. Study NCT03215095, is recruiting patients affected by histologically or cytologically
178 confirmed thyroid carcinoma of follicular origin (including papillary, follicular, Hürthle cell or poorly differentiated
179 subtypes and their respective variants) with diagnosis of recurrent and/or metastatic disease; the trial is designed to
180 assess the safety of administering the anti-PD-L1 antibody in combination with radioiodine (¹³¹I) upon
181 stimulation/preparation with recombinant human thyroid stimulating hormone. The only clinical trial which is
182 specifically recruiting patients affected with anaplastic thyroid cancer is study NCT02936102. In this study, patients
183 with triple negative breast cancer, non-small cell lung carcinoma, endometrial cancer and ATC will be treated with
184 FAZ053 as a single agent or in combination with PDR001, both drugs being anti-PD-L1 antibodies. Taken together

185 these evidences demonstrate that immunotherapy can represent a valid option for advanced and undifferentiated thyroid
186 cancer, but further dedicated trials are needed.

187 In summary, we conclude that VENTANA and R&D Systems antibodies are equally useful in revealing PD-L1 in ATC
188 cases and that normal thyroid as well as PTC or its insular variant do not show a detectable levels of expression. The
189 identification of PD-L1 in ATC may have direct therapeutic relevance to patients with refractory thyroid cancer.

190

191 **Compliance with Ethical Standards:**

192

193 **Conflict of interest:** Authors have no conflict of interests to declare.

194

195 **Ethical approval:** All procedures performed in studies involving human participants were in accordance with the
196 ethical standards of the institutional committee and with the 1964 Helsinki declaration and its later amendments or
197 comparable ethical standards.

198

199 **Informed consent:** Informed consent was obtained from all individual participants included in the study.

200

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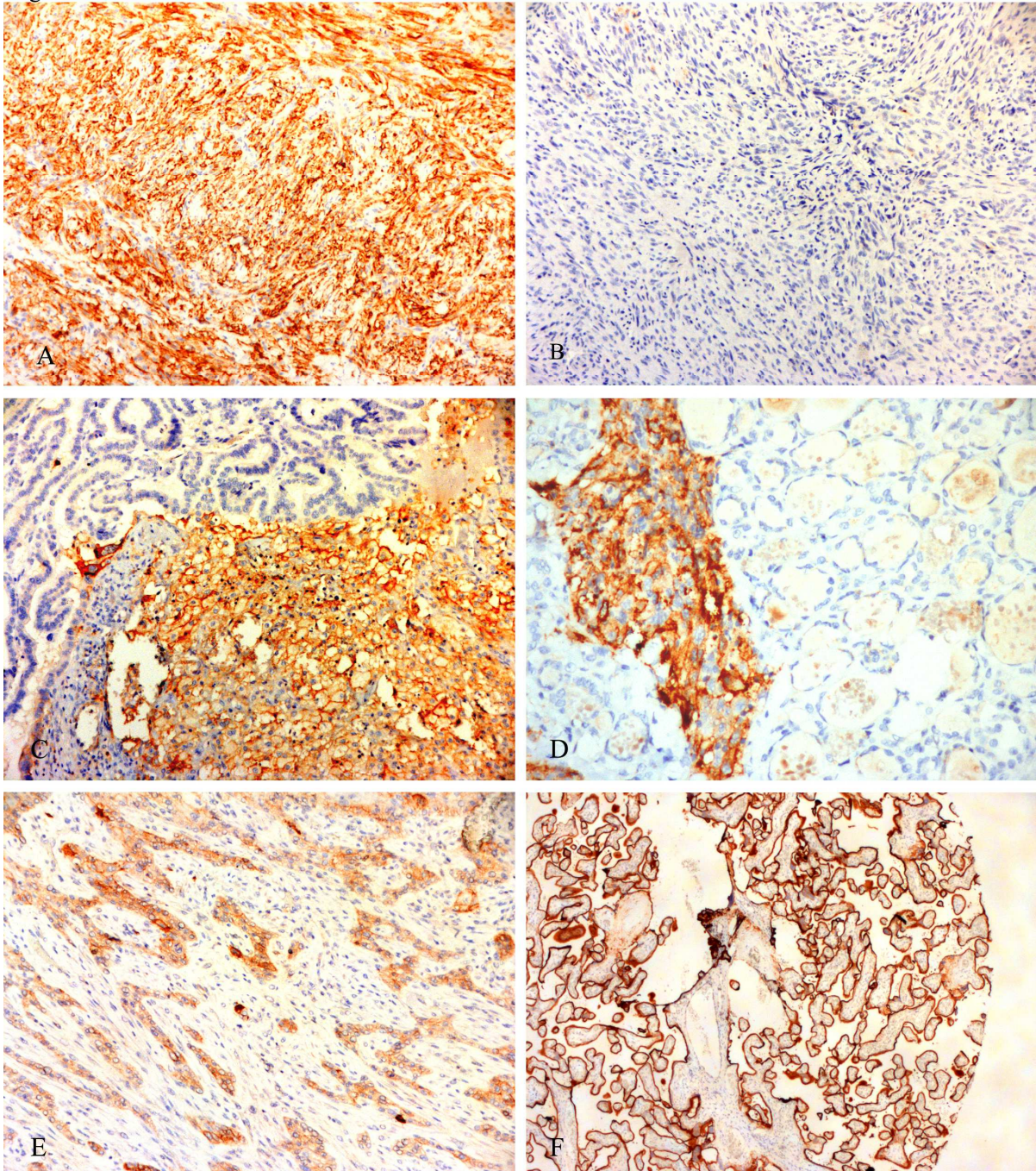
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Figure 1



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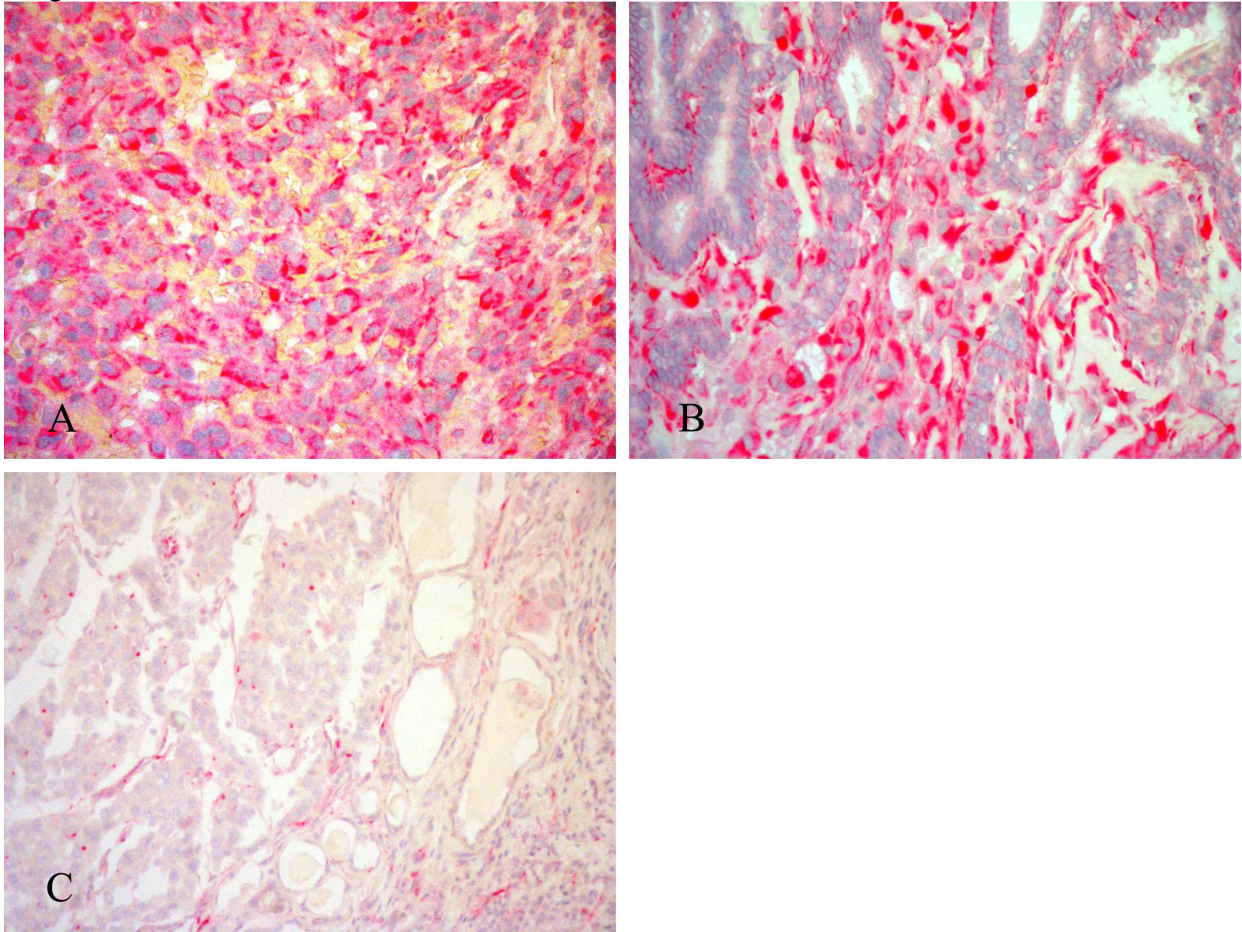
285 **Figure 1** A) ATC, VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. Strong and consistent positive
286 stain of neoplastic cells. Original magnification 40X.

287 B) ATC, VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. Negative case. Most of the cells are
288 completely negative, even though a minority of them (2-3%) show a weak positivity. Original magnification 40X.

289 C) Transition area between PTC and ATC, VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. In the
290 upper left corner PTC cells are not stained, whereas in the lower right corner ATC cells show a strong staining. Original
291 magnification 100X.

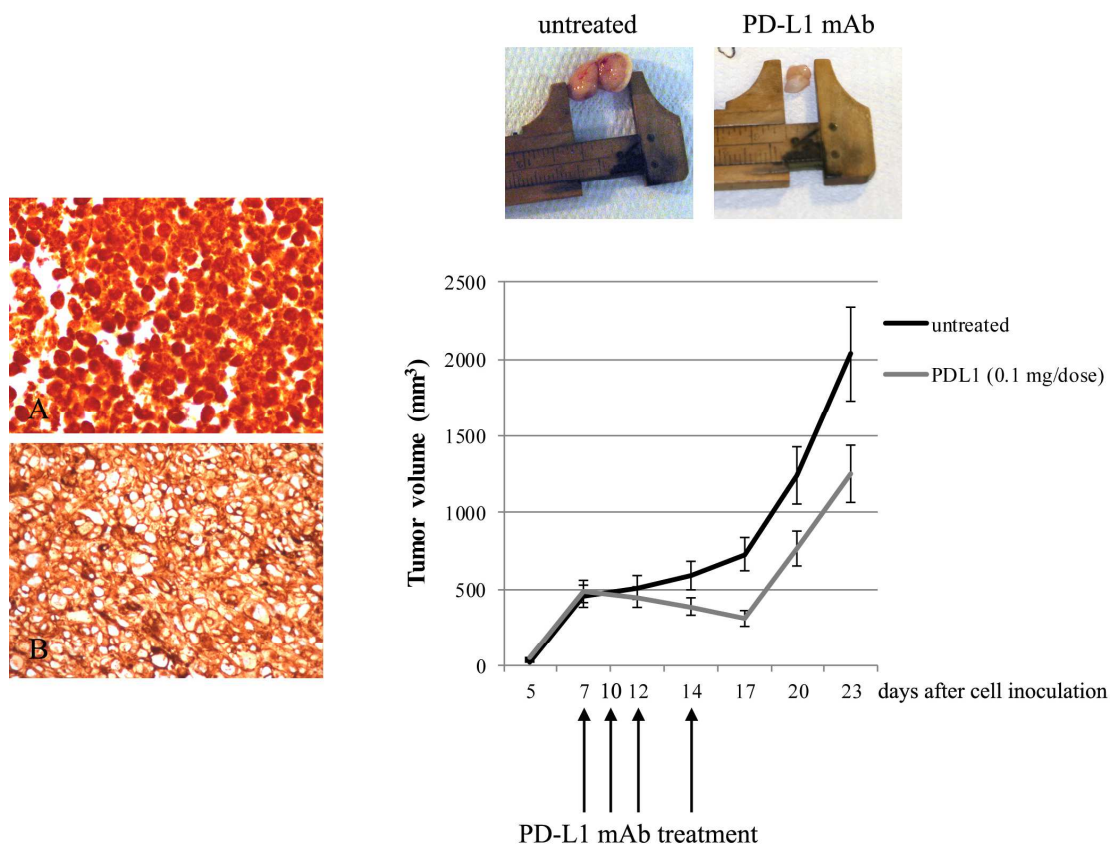
292 D) ATC, VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. An island of positively stained ATC cells
293 infiltrates an area of normal thyroid tissue which is unstained. Original magnification 100X.
294 E) ATC, VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. Cords of positively stained cancer cells
295 can be seen embedded within the unstained fibrous stroma. Original magnification 40X.
296 F) Placenta, VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. The positive control displays a strong
297 staining.

Figure 2



298 **Figure 2** A) ATC, anti-PD-L1 mAb R&D Systems (#MAB1561). ATC cells show a strong and diffuse positive
299 staining. Original magnification 100X
300 B) Transition area between PTC and ATC, anti-PD-L1 mAb R&D Systems (#MAB1561). An area of PTC (unstained
301 cells) is infiltrated by highly PD-L1 immunoreactive ATC cells in the stroma. Vascular endothelial cells are also
302 positive. Original magnification 100X
303 C) Poorly differentiated insular tumor, anti-PD-L1 mAb R&D Systems (#MAB1561). Scattered tumor cells show dots
304 of positive reaction. Endothelial cells are also PD-L1 immunoreactive. Original magnification 40X.
305
306

Figure 3



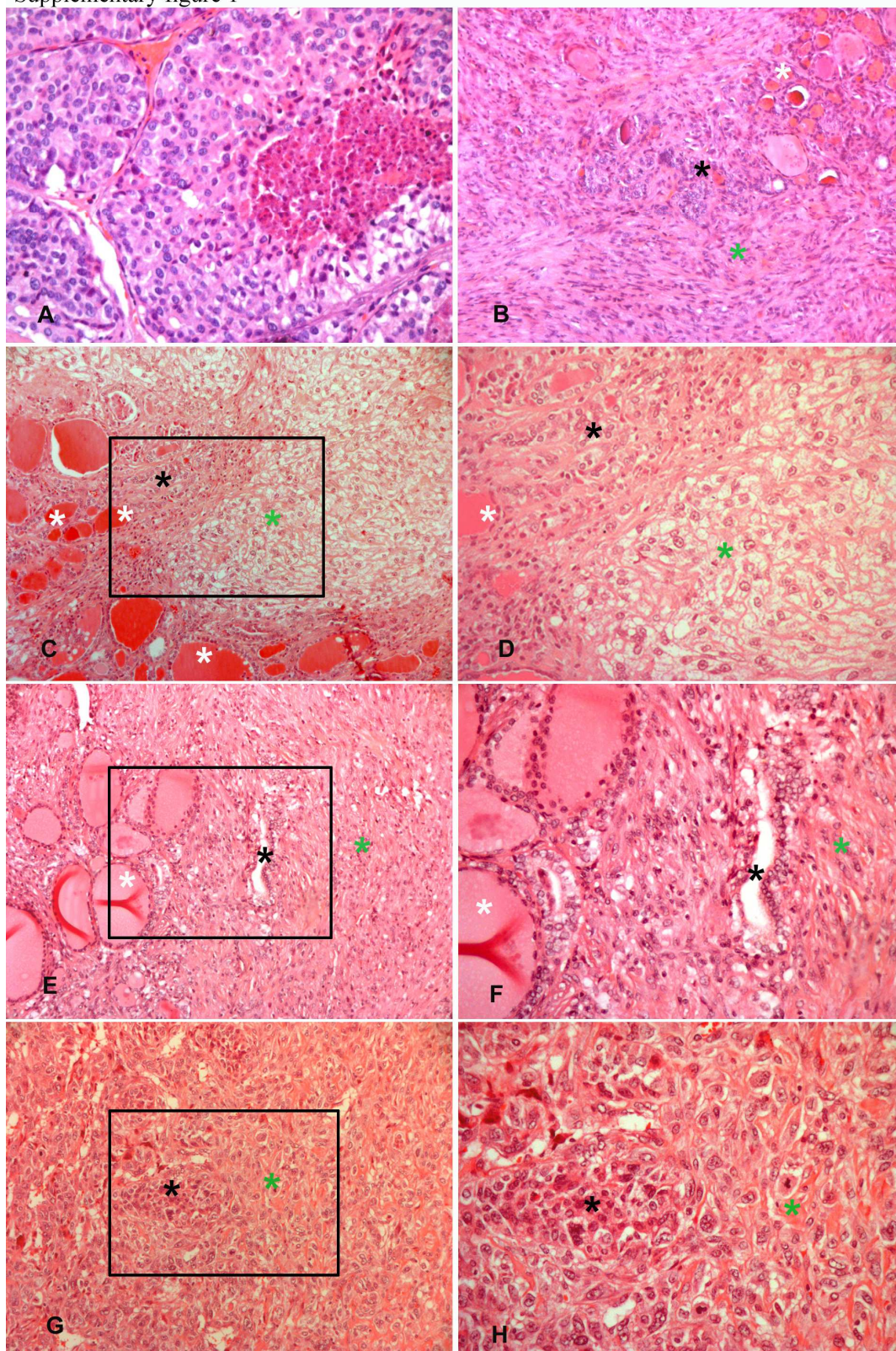
307

308 **Figure 3 A and B)** Strong positive PD-L1 immunoreaction in tumors from two different mice injected with human
 309 ATC cells employing the VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. **C)** Tumor volume
 310 calculated using the formula $\text{width}^2 \times \text{length} / 0.52$ and expresses as mm³. Days of immunotherapeutic treatment are
 311 indicate with arrows. Pictures of tumors explanted at day 23 are shown. Starting from day 17, tumors volume of treated
 312 and untreated animals show significant differences (* = $p < 0.05$).

313

314

Supplementary figure 1



Supplementary figure 1; Hematoxylin & Eosin stain; image representatives of the files object of the study.

A) Original magnification 200X; poorly differentiated thyroid carcinoma, insular variant; an area of necrosis in the center of an insula; neoplastic cells may sometimes be arranged in structures resembling thyroid follicles.

319 **B)** Original magnification 100X; the white asterisk marks normal thyroid follicles, the green asterisk undifferentiated
320 thyroid carcinoma, the black one an area of differentiated thyroid carcinoma.

321 **C)** Original magnification 100X; white asterisks mark normal thyroid follicles, the green asterisk undifferentiated
322 thyroid carcinoma, the black one an area of differentiated thyroid carcinoma.

323 **D)** Higher magnification (200X) of the framed region in C; Asterisks mark the same areas described in C.

324 **E)** Original magnification 100X; white asterisk on normal thyroid, on the right (black asterisk) differentiated thyroid
325 carcinoma and undifferentiated carcinoma (green asterisk).

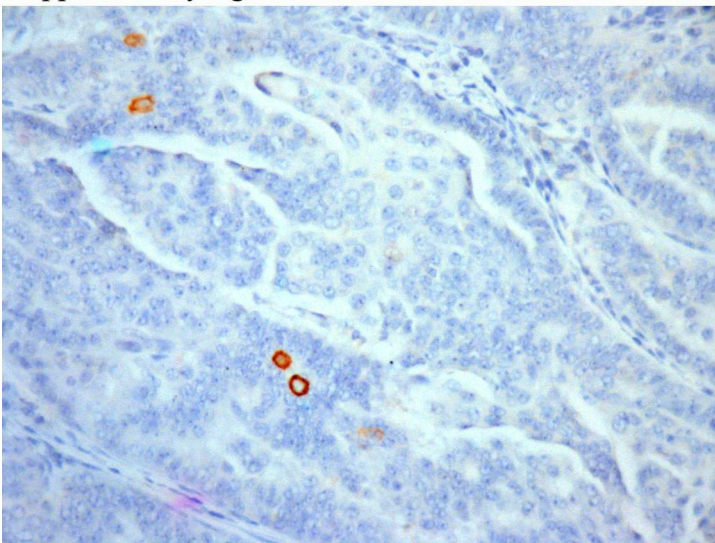
326 **F)** Higher magnification (200X) of the framed region in E; Asterisks mark the same areas described in E.

327 **G)** Original magnification 100X; the black asterisk marks poorly differentiated thyroid carcinoma with an insular
328 arrangement, while the green asterisk is on an area of undifferentiated thyroid carcinoma.

329 **H)** Higher magnification (200X) of the framed region in G; asterisks mark the same areas described in G. Close to the
330 green asterisk a mitotic figure.

331

Supplementary figure 2



332

333 **Supplementary figure 2**

334 Insular tumor, VENTANA PD-L1 (SP263) Rabbit Monoclonal Primary Antibody. Insular tumors may show scattered
335 PD-L1 immunoreactive cells.

336

337 **Table 1. Synopsis of patient's characteristics**

Case #	Sex	Age	Normal Thyroid Tissue Yes/No	Presence of differentiated TC
1	F	81	Y	No
2	M	85	Y	No
3	M	74	Y	PTC; insular carcinoma
4	F	79	Y	No
5	F	54	Y	PTC; insular carcinoma
6	F	78	Y	PTC; insular carcinoma
7	M	69	Y	PTC
8	F	82	Y	PTC; insular carcinoma
9	M	79	Y	PTC; insular carcinoma
10	F	87	Y	PTC; insular carcinoma
11	F	85	Y	PTC; insular carcinoma
12	M	76	N	No
13	M	78	Y	PTC; insular carcinoma
14	M	77	Y	No
15	M	60	Y	PTC; insular carcinoma
16	F	33	Y	PTC; insular carcinoma
17	M	72	Y	PTC; insular carcinoma
18	M	75	N	No
19	F	75	N	No
20	F	71	Y	PTC; insular carcinoma

338