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Research Article

RET/PTC3 translocation in a rare hemorrhagic brain metastasis of papillary thyroid cancer post Chernobyl radiation affects vessels ultrastructure

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ABSTRACT

Background: Slow progression and good prognosis are the usual characteristics of papillary thyroid carcinoma (PTC). The presence of brain metastases (0.4–1.2%) is suggestive of a worse prognosis. RET/PTC rearrangements were particularly prevalent in PTCs developed after Chernobyl nuclear accident.
Case description: A 50-year-old woman born in Slovakia, exposed to radiation resulting from the accident at the Chernobyl nuclear power plant, affected since 2017 by papillary thyroid cancer and in therapy at our hospital, experimented cerebral hemorrhagic metastasis. Biopsy analyses revealed a RET/PTC3 rearrangement, so our aim was to find possible morphological relation between hemorrhagic metastasis and RET/PTC3 translocation.
Results: Immunohistochemical analysis showed diffuse and intense positivity for VEGF in endothelial cells of the neoplasm' vascular network. Transmission electron microscopy images showed vessels with unorganized pattern and uneven diameters. In particular, metastasis endothelial cells (MECs) showed irregular shape and size, thickened cytoplasm and swelling of endoplasmic reticulum. MECs organized in irregular monolayers or multiple layers, surrounded by a thickened but unstructured extracellular matrix. Absence of strong junctional complexes among MECs resulted in a further weakened vessels wall.
Conclusion: RET/PTC3 translocation causes VEGF overexpression via STAT3 signaling cascade and the increased amount of VEGF adds to the greater amount of VEGFRs expressed by MECs. Our ultrastructural investigation show that this condition creates a massive growth of altered vessels prone to bleeding. The clinical significance of our study consists in alert oncologist and surgeons on possible arising of hemorrhagic brain metastases in patients with PTC and RET/PTC3 translocation exposed to ionizing radiation as people living in areas caught up in Chernobyl or Fukushima disasters.

1. Introduction

Thyroid carcinomas represent 3–4% of all human tumors [1]. Thyroid carcinoma is classified in four histotypes: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC) and anaplastic thyroid carcinoma (ATC), being PTC the most frequent histotype (80%) [2]. Studying genetic alterations involved in thyroid cancer pathogenesis, the rearranged during

Abbreviations: PTC, Papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; MTC, medullary thyroid carcinoma; ATC, anaplastic thyroid carcinoma; MEC, Metastasis endothelial cell; EM, Transmission electron microscopy; LM, Light microscopy

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transfection (RET) proto-oncogene was found playing a role in PTC development. A chromosomal rearrangement (named RET/PTC) [3] activates the gene. Multiple downstream pathways promoting cell growth, proliferation, survival and differentiation are stimulated by RET activation [4]. RET/PTC3 rearrangement consist in RET tyrosine kinase domain fusion with the nuclear receptor co-activator 4 gene (NCOA4), was described in PTC [5]. Exposure to ionizing radiation is the main factor in development of RET/PTC rearrangements [6–8], being these rearrangements particularly prevalent in PTCs developed after Chernobyl nuclear accident [9–12].

Normally, the PTC has slow progression and good prognosis. The distant metastases (their main locations are lungs and bones) are very uncommon, accounting from 0.1% to 5% of PTCs [13] and they are suggestive of a worse prognosis. Brain metastases are even rarer, their reported incidence is 0.4–1.2% [14], this let us to study, by immunohistochemistry, light and transmission electron microscopy, a patient of our Hospital experimented cerebral hemorrhagic metastasis in a radio-induced PTC (post-Chernobyl) expressing RET/PTC3 rearrangement. Considering the hemorrhagic feature of the metastatic lesion, our aim was to study its vessel network ultrastructure (no ultrastructural studies concerning capillaries in tumor lesions of this type are present in literature) in order to find possible morphological alterations related to hemorrhagic pattern and RET/PTC3 rearrangement. Such a correlation could be clinically useful in alert oncologist and surgeons on possible arising of hemorrhagic brain metastases in patients with PTC and RET/PTC3 translocation exposed to ionizing radiation.

2. Materials and methods

2.1. The patient

A 50-year-old woman, born in Slovakia, exposed to radiation resulting from the accident at the Chernobyl nuclear power plant, affected since 2017 by papillary thyroid cancer, tall cell variant, probably radio-induced in nature, for which she underwent to a thyroidectomy with left radical neck dissection. The histological examination showed the presence of a 21 mm lesion, at the level of the left thyroid lobe, identified as papillary thyroid carcinoma, tall cell variant, with infiltration of the thyroid capsule, striated muscle tissue and neoplastic vascular embolization. In April 2018, the patient also underwent radiometabolic treatment with Iodine-131. During the clinical and radiological follow-up, post-therapeutic body scintigraphy identified areas of pathological collection in the cervical area bilaterally, followed by further bilateral radical neck dissection. In May 2019, she underwent a new cycle of radiometabolic therapy. In June 2019, the last staging with PET/TC total body was performed, this exam documented pathological fixation at the cervical-mediastinal, cervical-dorsal, retrosternal, pericardial and intracranial levels. It is thus classified as stage II TNM (T2m, N1b, M1). Considering the presence of pathological uptake also in the brain, the patient underwent brain MRI scan with gadolinium, gold standard for the identification of any intracranial metastasis. MRI thus documented the presence of a left pre-central rounded formation with blood content of about 1.5 cm (Fig. 1a, b). The patient preoperative neurological status consisted in the absence of neurological impairments. Complete deficiency of abduction and elevation from left upper limb, resulted of previous left radical neck dissection. In July 2019, the patient underwent surgery to remove the left pre-central hemorrhagic lesion (Fig. 1c, d). In the post-operative, the patient was mobilized in the first day, in the absence of further neurological deficits. The histological examination confirmed the metastatic nature of the lesion from papillary thyroid carcinoma, tall cell variant, identifying with the help of the real time PCR the translocation RET/PTC3.

The study was approved by the Institutional Ethics Board and adhered to the tenets of the Declaration of Helsinki.

2.2. Immunohistochemical analysis

Briefly, the sampling areas of thyroid neoplasm were achieved from formalin-fixed specimens. Then, the tissues areas were embedded in paraffin to obtain a formalin-fixed paraffin embedded tissue pieces (FFPE), that were further cut into 3 μm thick tissue sections and assembled on microscope slides. The sections were finally deparaffinized and stained with hematoxylin and eosin to highlight both the architecture and the features of the neoplastic cells. According to the laboratory practice, we performed immunohistochemistry on paraffin slide. After rehydration and antigen retrieval (15 min in citrate buffer pH 6) slide was incubated with VEGF antibody (clone ABS82) overnight at 4 °C. After washing in phosphate saline buffer, slide was incubated with secondary universal antibody (Scytek Laboratories, West Logan) and immunoreactions was detected with diaminobenzidine (Vector Laboratories, California).

We analyzed the expression of the immunohistochemical marker by the endothelial cells of the vascular structures of the neoplasm.

2.3. Light and transmission electron microscopy protocols

Samples were fixed in 2.5% glutaraldehyde in PBS 0.1 M pH 7.4 at least for 48 h at 4 °C and then rinsed with PBS. Samples were then post-fixed with osmium tetroxide 1.33% (Agar Scientific, Stansted, UK) for 2 h and washed with PBS for 20 min in order to remove osmium tetroxide residuals. Specimens underwent dehydration steps in ascending ethanol series (30%, 70%, 95%, 100% v/v × 3). Ethanol substitution with propylene oxide was performed (BDH Italia, Milan, Italy). Samples were embedded in a mixture 50:50 propylene oxide and epoxy resin Agar 100 (SIC, Rome, Italy) overnight at 25C° under chemical fume hood. Finally, samples were embedded in Agar 100 resin, put in a stove at 60C° for 48 h. Semithin sections (1 μm thick) were collected on glass slides, stained blue by Azur II, in order to perform light microscopy (LM) observations a light microscope (Carl Zeiss Axioskop-40, Zeiss, Germany). LM observation of 1 μm thick epoxy resin semithin sections allows imaging at high magnification (1000X) and with excellent resolution [15]. Then, ultrathin sections (80–90 nm), for transmission electron microscopy (TEM) observation, were cut using an ultramicrotome (Leica EM UC6, Vienna, Austria). Ultrathin sections were collected on 100-mesh copper grids (Assing, Rome, Italy) stained with Uranyless© solution and lead citrate [16,17]. Imaging was performed using a transmission electron microscope set with an accelerating voltage of 60 kV (Carl Zeiss EM10, Thornwood, NY). Images were acquired with a CCD digital camera (AMT CCD, Deben UK Ltd, Suffolk, UK).

3. Results

3.1. Immunohistochemical observations

Sections from the metastasis, stained for VEGF, showed a marked increasing in vascular structures, moreover endothelial cells showed diffuse and intense positivity for VEGF (Fig. 2).

3.2. Light microscopy observations of semithin sections

Images of semithin sections showed a very high degree of vascularization. Vessels were unevenly distributed in the metastatic tissue. Absence of normal hierarchical organization was found, together with patent and not-patent vessels (Fig. 3). Areas with clustered vessels were observed (Fig. 3a, b, c); as well as areas completely devoid of them (Fig. 3b). Vessels were represented by almost unusually large capillaries (8–10 time larger than normal) with hyalinized wall (Fig. 3c, d, e). In each section, several vessels appeared filled not only with blood cells but also with cancer cells (Fig. 3d). Endothelial cells hyperplasia was often found, being present in a single vessel section numerous

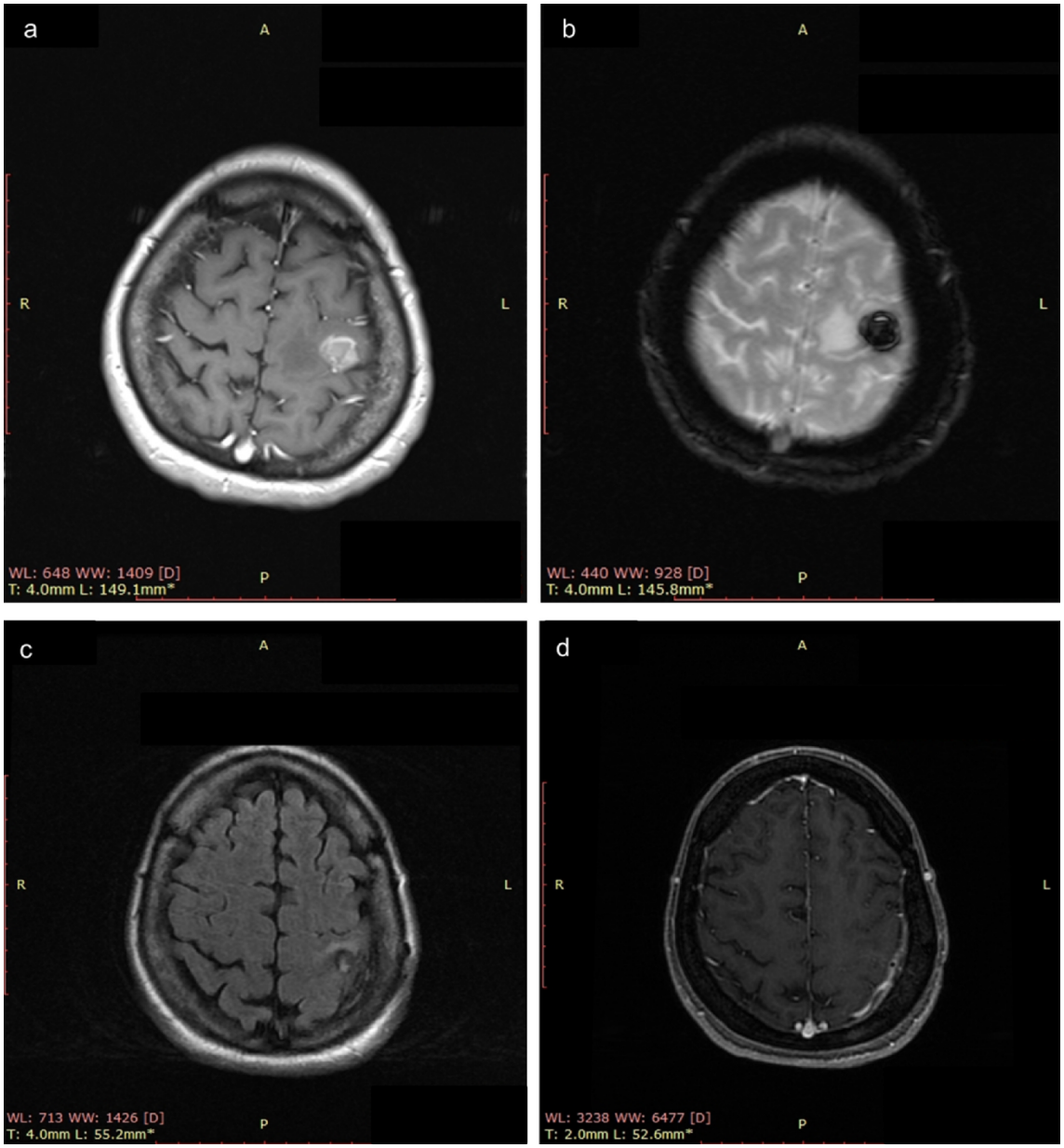

Fig. 1. Pre-operative (a, b) and post-operative (b, c) MRI. a) axial, T1-weighted contrast-enhanced magnetic resonance imaging demonstrating a left pre-central lesion suggestive for metastasis, showing a not homogeneous contrast-enhancement surrounded by perilesional edema; b) axial, T2-weighted gradient-echo magnetic resonance imaging demonstrating the same left pre-central lesion with hemosiderin content, due to recent bleeding, surrounded by perilesional edema. c) axial, T2-weighted FLAIR magnetic resonance imaging demonstrating the metastasis total resection, associated with significant reduction of perilesional edema; d) axial, T1-weighted contrast-enhanced magnetic resonance imaging acquired post-operative, demonstrating the metastasis total resection.

endothelial cell nuclei. Some capillaries showed an irregular endothelial monolayer, some others presented a double layer (Fig. 3e). The perivascular stroma of the metastatic tissue appears edematous and numerous extravasated blood cells were visible inside the extracellular matrix (Fig. 3 d).

3.3. Transmission electron microscopy observations

Images of vessel wall showed an abnormally thickened perivascular extracellular matrix and an irregularly multilayered basal lamina, hyalinized at light microscopy observation (Fig. 4a,b).

Metastasis endothelial cells (MECs) appeared thickened, their nuclei had irregular border and sometimes indentations (Fig. 4b). Pericytes were observed surrounding blood vessel, but they formed abnormally loose associations with MECs (Fig. 4a, b, c). Very often endothelial cells were multilayered, their cytoplasm contains vacuoles, damaged mitochondria, vesicles (Fig. 4a, b, c). Dilated endoplasmic reticulum unravels from external nuclear membrane (the space between nuclear membranes itself appear dilated); some apoptotic bodies (0.8–5.0 μm) were found (Fig. 4c, d, e, f; 4c, d). Endothelial cell junctions appear loose; rare tight and adherens junctions were present. Unlike, numerous fenestrations and spaces among endothelial cells were observed (Fig. 5a, b).

Higher magnifications showed details of irregular basal lamina, with collagen bundles circularly and longitudinally oriented. Fibrin and collagen fibers mixed up, creating a disorganized perivascular space (Fig. 5a,b). Images at higher magnifications clarified the details of cytoplasmic vesicle content. Cytoplasm contained exosomes of 50–100 nm in diameter, microvesicles of 150–200 nm in diameter and multivesicular bodies filled with exosomes (Fig. 5c, d).

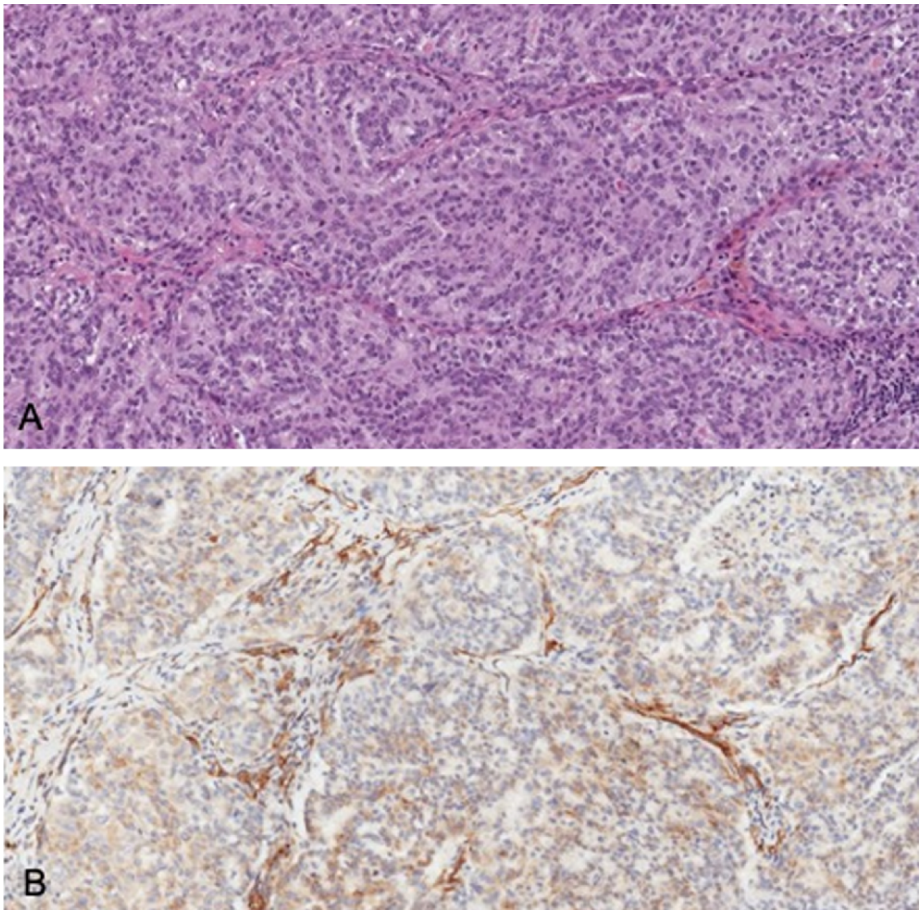

Fig. 2. Papillary thyroid carcinoma, immunohistochemical analysis of vascular network. A. hematoxylin-eosin stain, magnification 200X. B. Endothelial cells showed diffuse and intense positivity for VEGF, immunohistochemical stain, magnification 200X.

4. Discussion

Radiation can cause cancer even many years later, but can also be used as a therapy to cure the cancer themself [18,19]. Cancer development risk after radiation exposure begin to decline about 30 years after exposure, even if it remains still elevated at 40 years [20,21]. As it shows, unfortunately, what happened to our patient. She was a young woman of 19 years old in 1986, and she suffered for thyroid cancer after 30 years, in 2016. Her neoplasm was a papillary thyroid cancer, tall cell variant, with the RET/PTC3 translocation. The association of RET/PTC3 with radiation-induced papillary thyroid cancer is well established, particularly in the solid variant [22,23] and the same mutation has also been associated with the Tall Cell Variant we found in our patient[24]. She experimented cerebral hemorrhagic metastasis, a very rare finding, so our aim was to study vessel network ultrastructure of metastatic lesion by immunohistochemical analysis and TEM observations, being absent in literature immunoistochemical and ultrastructural studies concerning capillaries in tumor lesions of this type, in order to find possible morphological alterations related to hemorrhagic pattern and RET/PTC3 rearrangement. The existence of this correlation could be clinically useful to alert oncologist and surgeons on possible arising of hemorrhagic brain metastases, in patients with PTC and RET/PTC3 translocation exposed to ionizing radiation.

RET/PTC3 rearrangement is present in the more aggressive PTCs variants and has a role in metastatic spread [25,26]. RET activate different signaling cascades and interacts with many molecules, among whom there is the signal transducer and activator of transcription-3 (STAT3). It is a cytoplasmic transcription factor involved in several mechanisms during in vitro cultured cell transformation [27,28]. RET/PTC correlates with STAT3 and promotes its activation by phosphorylation of STAT3 tyrosine 705 residue [29], this represent one of the critical signaling pathways that regulate specific genes, such as VEGF. VEGF is an important trigger of angiogenesis, it plays a role in different malignant cancers proliferation and spread. In adults and children, VEGF overexpression correlates with PTC size [30], higher VEGF expression associates with metastasis [31]. A statistically significant relationship, between VEGF expression and blood vessel content in PTCs, is demonstrated by Vieira et al. [32], because endothelial cells of cancer blood vessels expresses VEGFRs. In our patient, we found endothelial cells expressing diffuse and intense positivity for VEGF (Fig. 2), metastasis blood vessels with unorganized pattern, arising from absence of vessel hierarchical branching present in the normal vasculature [33]. We observed MECs forming irregular monolayers or multiple layers disorganized and loosely connected as previously reported in cancer diseases [34]. We found that their basement membranes had variable thicknesses, unlike normal blood vessels [35]. Pericytes were observed surrounding metastasis blood vessels, but they form abnormally loose associations with MECs, as reported by Baluk et al. [36], and results in cancer blood vessels leakiness. The more impressive observation was the presence of vessel with uneven diameters, (this is due to compression of the immature vessel wall by tumor cells) sometimes exhibiting chaotic blood flow, sometimes not patented at all. Examining MECs we noted that they are irregular in shape and size, with thickened cytoplasm and endoplasmic reticulum dilatation. Few intercellular junctions are present (very scarce adherens and tight junctions); this condition allows both the intravasation of cancer cells, favoring metastases formation [37], and the process of extravasation, as hypothesized in the pathogenesis of chronic subdural hematoma enlargement [38].

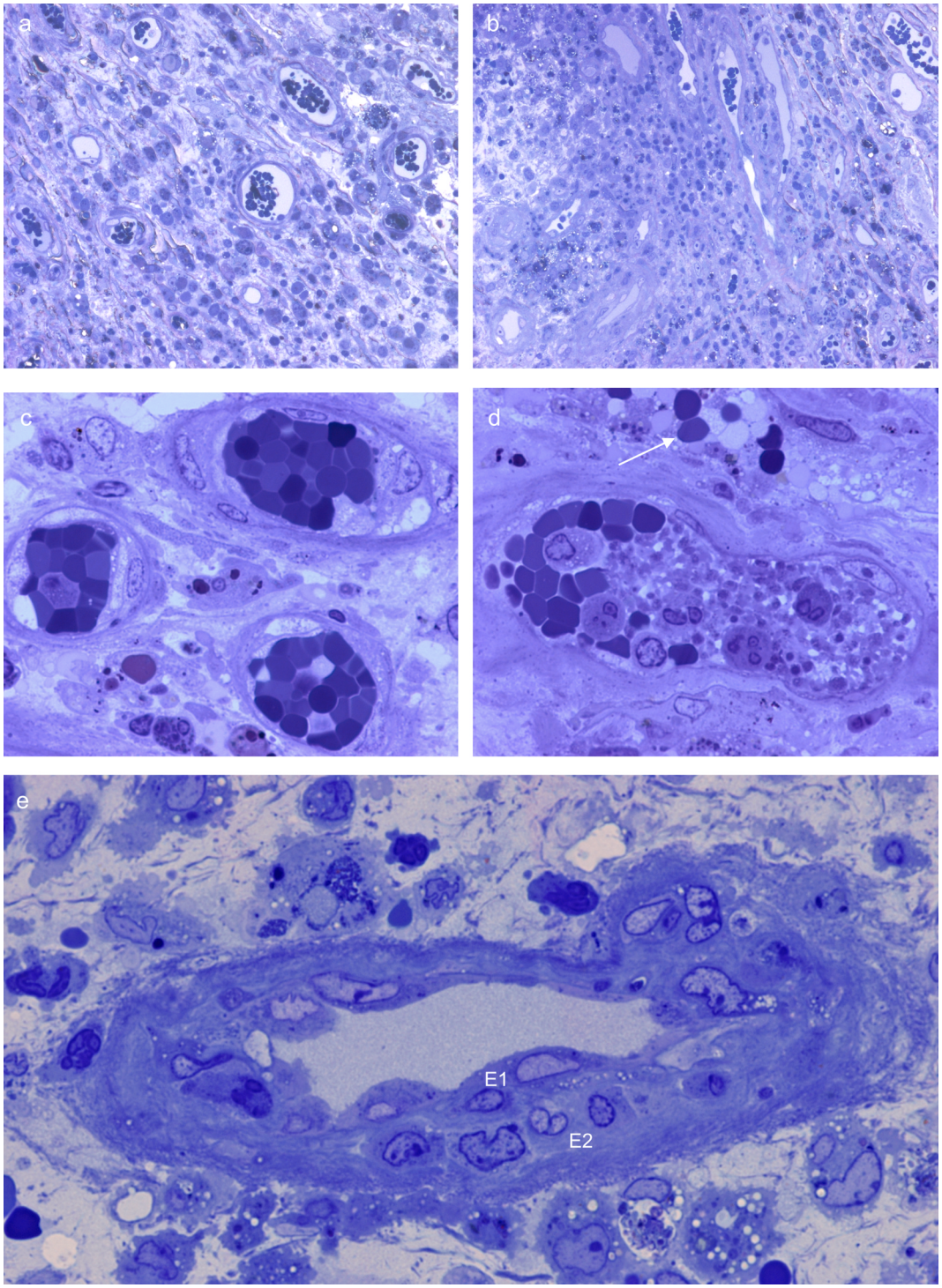

Fig. 3. Blood vessels anomalies at light microscopy. a) Numerous capillaries in transverse section are visible. Some vessels show blood cells inside them, others don't, 400X. b) Numerous capillaries in longitudinal section are visible, they show irregular and tortuous lumen, 400X. c) Unusually large capillaries, with double layer of endothelial cell (right part of picture) filled with blood cells 1000 X. d) Hyalinized vessel filled with red blood cells, granulocytes, platelets and cancer cells. Extravasated blood cells were visible inside the extracellular matrix (arrows) 1000 X. e) Large capillary with hyalinized wall, and double endothelial lining (E1, E2) 1000X. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cancer endothelial cells are different from normal endothelial cells [39,40] in their responsiveness to VEGF [41]. A VEGF autocrine loop in cancer endothelial cells confers at least partial resistance to serum starvation, usually not observed in normal endothelial cells [41,42]. Compared with normal endothelial cells, cancer endothelial cells were more responsive to VEGF [41]. In addition, cancer endothelial cells originate also from differentiation of cancer stem cells under specific hypoxia conditions via VEGFR-2 [43].

Moreover, cancer stem cells might also generate pericytes to support vessel function and tumor growth [44]. For what reported above, we can state that ultrastructural modifications of blood vessel in this rare hemorrhagic brain metastasis of papillary thyroid cancer, tall cell variant, post Chernobyl radiation are very likely due to the presence of a RET/PTC3 translocation. In fact, RET/PTC3 intensify VEGF

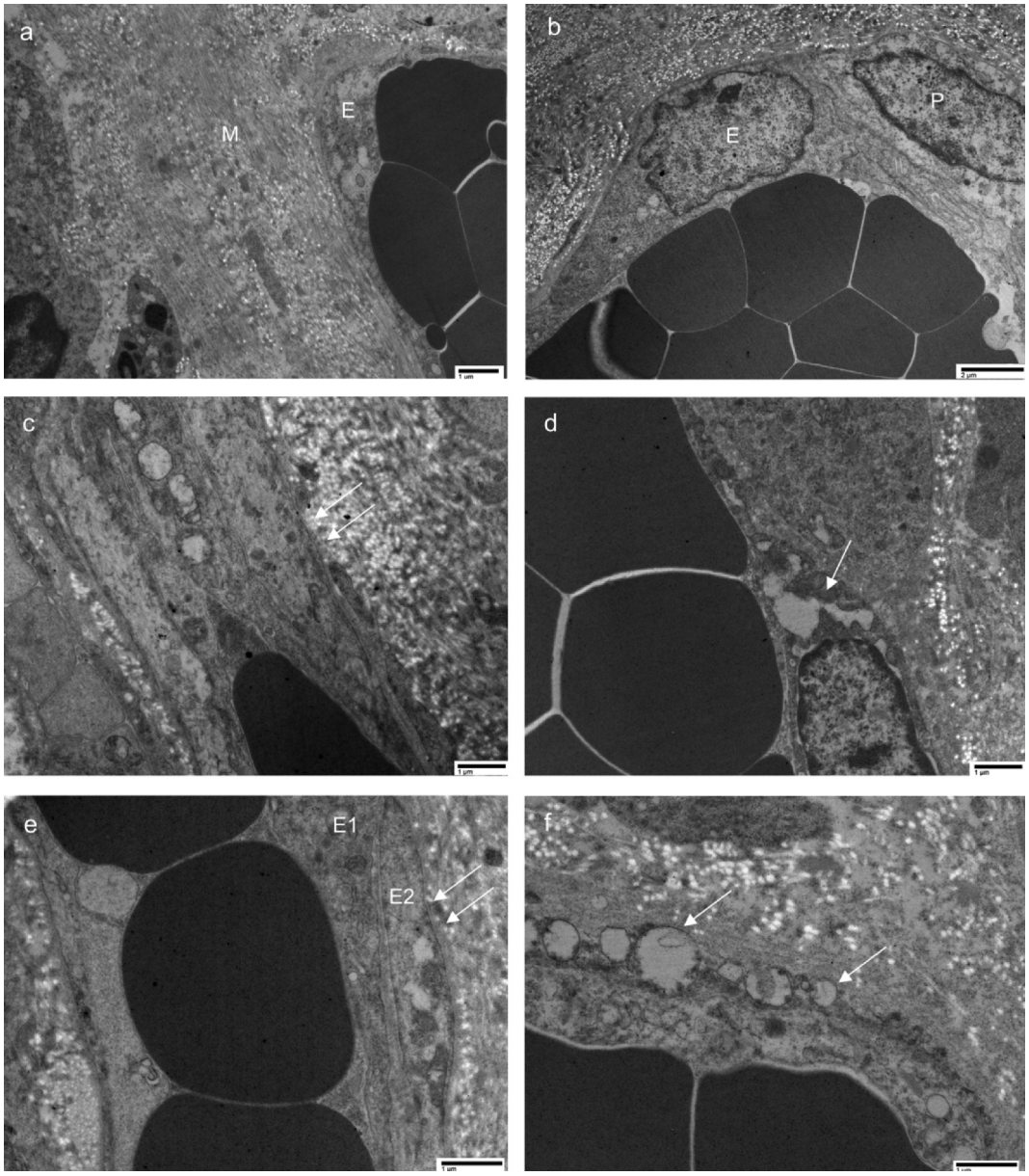

Fig. 4. Metastatic vessel basal lamina and endothelial cells anomalies at transmission electron microscopy. a) Vessel basal lamina appears hyalinized i.e. abnormally thicker, with collagen bundles circularly and longitudinally oriented. Among collagen fibers fibrin is dispersed and perivascular space appear edematous, (E = vessel endothelium, M = extracellular matrix) 14.000X. b) Endothelial cell (E, on the left) had larger amount of cytoplasm than usual, shows elongated nucleus with irregular border and sometimes indentations. A pericyte (P, on the right) loosely associated with endothelial cell is visible, 10.900X; c) Tangential section of a capillary vessel; the multilayered structure of the capillary wall is evidenced (arrows). Apoptotic bodies and an abnormally thicker basal lamina, with collagen bundles circularly and longitudinally oriented, is visible 16.800X. d) An endothelial cell (E) with dilated endoplasmic reticulum (arrow) unraveling from external nuclear membrane layer, 16.800X. e) Endothelial cells overlap forming a bilayer (E1, E2). They are surrounded by a thickened basal lamina (arrow), 21.300X. f) An endothelial cell with numerous vesicles inside the cytoplasm and beneath the external side of the cell (arrows). Basal lamina appears as thickened structure, with some collagen bundles circularly and longitudinally oriented and amorphous material dispersed in the perivascular space, 21.300X.

overexpression via STAT3 signaling cascade, the increased amount of VEGF adds to the greater amount VEGFRs expressed by MECs, creating an explosive growth of altered blood vessels, which, in turns, caused bleeding. The immunohistochemical analysis of metastasis MECs showed how they were diffusely and intensely positive for the VEGF antibody. This finding allowed us confirming the activation of the molecular pathway we hypothesized, excluding other possible pathways, as NF-κB and NIK, observed in association with RET/PTC3 mutations [45]. Not least because NF-kB-regulated processes are mainly inflammation (as in chronic and autoimmune disorders), immunity (e.g., immunodeficiencies) and cell proliferation and survival (e.g., cancer). Activation of this pathway is not related (up to now, for what is in our knowledge) with blood vessel ultrastructural alteration or VEGF increased expression [46]. The possible pathophysiological role of VEGF was also previously hypothesized in an interesting immunochemical study in metastatic brain tumor-associated intracerebral hemorrhage [47]. Our observations, obtained in this rare metastatic lesion, showed the ultrastructural basis for this pathophysiological process.

For what concern the observed rich cytoplasmic vesicles content of MECs, they are mainly exosomes (30–100 nm in diameter), microvesicles (100 nm-1 μm) and some apoptotic bodies (0.8–5.0 μm) [48,49]. Extracellular vesicles transport proteins (membrane or cytosolic), lipids, DNA, mRNAs and miRNAs [50–54]. Microvesicles

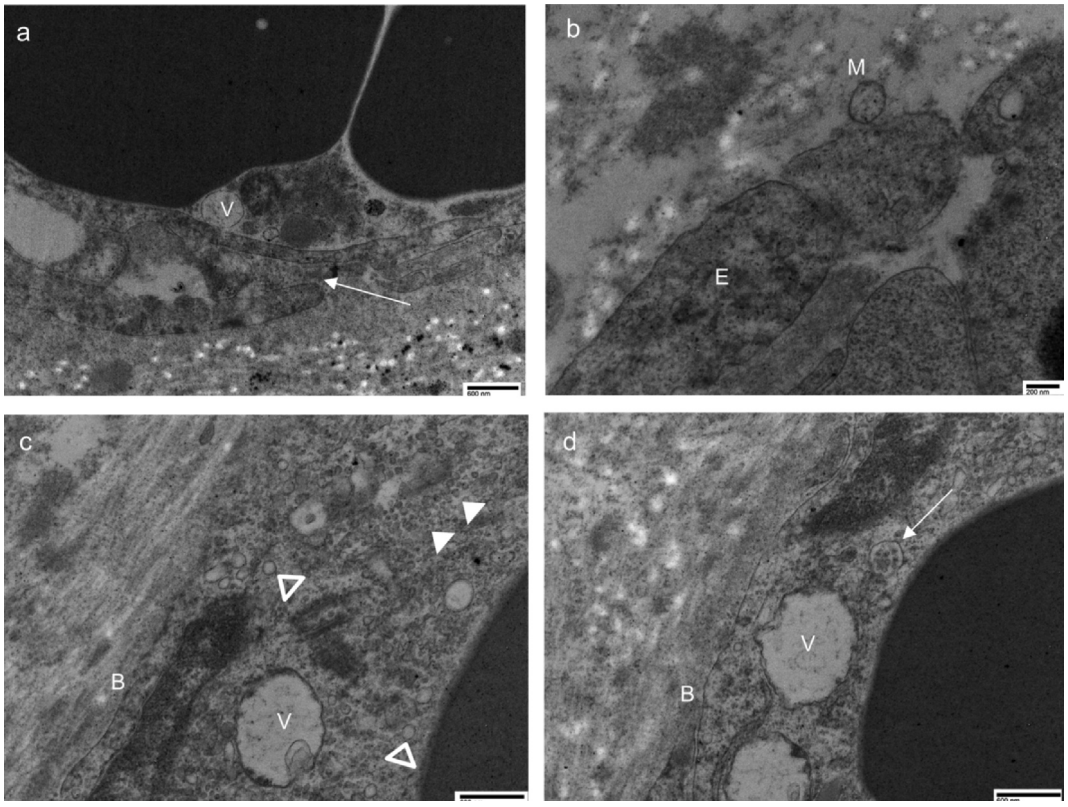

Fig. 5. Metastatic endothelial cells anomalies at transmission electron microscopy. a) Fenestrations and spaces (arrow) between endothelial cells were observed; they are filled by amorphous material, as well as the microvesicles (M) present in the capillary lumen, 28.600X. b) At high magnification, it is evident the presence of microvesicles (M) outside and exosomes (E) inside the endothelial cell, note the absence of the basal lamina56.300X. c) Endothelial cell cytoplasm appears rich in vesicles, mainly of 50 nm (arrowhead) and 100–120 nm in diameter (empty arrowhead), vacuoles (V); an irregular basal lamina (B) is evident, with some collagen bundles and amorphous material in the perivascular space, 35.900X. d) Note the presence of a multivesicular body (arrow) and two vacuoles (V) in the cytoplasm of endothelial cell. An irregular basal lamina (B) is evident, 35.900X.

secreted by cancer cells and containing EGFR reprogram endothelial cells, prompting them to express and respond to VEGF in an autocrine manner [55].

Moreover, it must be considered that very small quantities of intracellular VEGF are expressed by normal endothelial cells in vivo, activate VEGFR-2, and are required for vascular homeostasis. An alteration in this balance leads to micro-vasculature destabilization which, in turn, cause hemorrhage [56].

In conclusion, our ultrastructural study, the first on a hemorrhagic brain metastasis of PTC tall cell variant post Chernobyl radiation with RET/PET3 rearrangement, shows morphological correlation between vessels ultrastructural alterations, RET/PET3 rearrangement and the hemorrhagic clinical presentation of the metastasis. Exposure to ionizing radiation can have consequences even decades later, it is therefore important to keep the attention of oncologists and surgeons high on the possibility that people from areas affected by nuclear accidents, as Chernobyl or Fukushima, may develop PTC tall cell variant with RET/PTC3 translocation and hemorrhagic brain metastases.

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CRediT authorship contribution statement

Michela Relucenti: Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Pietro Familiari:** Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Giorgia Iacopino:** Methodology, Writing - review & editing. **Placido Bruzzaniti:** Methodology, Writing - review & editing. **Selenia Miglietta:** Software, Data curation, Writing - review & editing. **Maurizio Salvati:** Resources, Writing - review & editing. **Xiaobo Li:** Conceptualization, Validation, Writing - review & editing. **Rui Chen:** Conceptualization, Validation, Writing - review & editing. **Giancarlo D'Andrea:** Resources, Writing - review & editing. **Alessandro Frati:** Resources, Writing - review & editing. **Cira Di Gioia:** Formal analysis, Investigation, Writing - review & editing. **Angelina Pernazza:** Formal analysis, Investigation, Writing - review & editing. **Carlo Della Rocca:** Investigation, Writing - review & editing. **Giuseppe Familiari:** Resources, Data curation, Supervision, Project administration, Funding acquisition, Writing - review & editing. **Antonio Santoro:** Conceptualization, Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.inat.2020.100889.

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