Cytoplasmic Staining of OCT4 Is a Highly Sensitive Marker of Adrenal Medullary–derived Tissue

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Abstract: OCT4 immunostaining has become an essential resource in diagnosing germ cell neoplasia. OCT4 is a transcription factor with a characteristic nuclear staining pattern specific to germ cell neoplasms. Our institution has observed that paraganglionic tissue consistently displayed intense cytoplasmic staining by utilizing monoclonal OCT4 antibody, and we intended to determine whether OCT4 could provide additional diagnostic utility in adrenal tumors. We used monoclonal and polyclonal OCT4 antibodies for comparison of staining patterns and intensities. Thirty-eight pheochromocytomas (8 metastatic), 22 adrenal cortical carcinomas (2 metastatic), 15 metastatic tumors to the adrenal glands, and 10 normal adrenal glands containing cortical and medullary tissue were immunostained with OCT4. A 4-tier system (0 to 3), for recording intensity and extent of cytoplasmic staining, was used. All 30 primary pheochromocytomas displayed strong and diffuse (3 + 3) cytoplasmic immunoexpression. Six of 8 metastatic pheochromocytomas showed strong immunoexpression (3 + 3), whereas the remaining 2 showed moderate intensity (2 + 3). All 22 adrenal cortical carcinomas, including metastatic cases, were completely negative. Only 2 metastatic tumors to the adrenal gland showed weak, cytoplasmic positivity: a small cell carcinoma and a Merkel cell carcinoma. Controls stained in an appropriate nuclear manner. Immunoelectron microscopy demonstrated the antibody interacting with neurosecretory granules. To our knowledge, the cytoplasmic expression of OCT4 in adrenal medulla and pheochromocytoma has not been specifically studied. The goal of this study is to analyze the immunoreactivity of adrenal cortical carcinoma and pheochromocytoma to OCT4 and determine the sensitivity and specificity of this particular staining pattern and to compare monoclonal and polyclonal antibodies.

Key Words: OCT4, pheochromocytoma, adrenal cortical carcinoma, immunohistochemistry

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OCT4 (OCT3/4, POU5F1) is a POU-domain, octamer-binding transcription factor that is involved with regulation of downstream targets, such as *Nanog*, which are involved with maintaining the pluripotency of embryonic stem cells (ESC), adult stem cells, and germ cells.[1–5] Disruption of the *OCT4* gene, located on chromosome 6p21.3, has been shown to be essential for the self-renewal of ESCs in mouse development.[6] In recent times, supportive evidence has accumulated indicating that *OCT4* is capable of encoding 2 different isoforms, the nuclear-localized OCT4A and the cytoplasmic-localized OCT4B, of which OCT4A is the isoform with known biological activity.[3,4,7]

The use of OCT4 as a diagnostic marker began to gain interest after the work by Looijenga et al,[8] which showed that OCT4 was specific for cells with pluripotent potential, particularly seminoma and embryonal carcinoma components of germ cell tumors. Further work at our institution, with a much greater number of cases, demonstrated the great sensitivity and specificity OCT4 has in seminoma, embryonal carcinoma, and intratubular germ cell neoplasia.[1,2,6] In addition, OCT4 has proven to be the most useful marker in identifying metastatic germ cell tumors and primary germ cell tumors (containing seminoma or embryonal elements) arising outside the testes or ovaries.[6,9–11]

As a high-volume germ cell tumor center, our institution sees a large number of patients with metastatic germ cell tumors and utilizes OCT4 staining regularly. We noticed that paraganglionic tissue removed during retroperitoneal lymph node dissection procedures stained intensely for OCT4 in an unusual, cytoplasmic manner. Intrigued by these findings and the growing evidence that OCT4 expression is not strictly limited to ESCs and germ cells,[12–15] an analysis of the staining pattern of OCT4 utilizing 2 separate antibodies—a monoclonal (cell Marque) and a polyclonal (Santa Cruz)—in adrenal cortical carcinoma (ACC), metastatic tumors to the adrenal gland of nonadrenal origin, and in normal adrenal gland was also performed to strengthen our findings. In addition, ultrastructural studies with immunoelectron microscopy were utilized to elucidate the antigen-binding site of the OCT4 antibody.

MATERIALS AND METHODS

Thirty cases of primary pheochromocytoma, 8 cases of metastatic pheochromocytoma, 22 cases of ACC

(2 metastatic), 10 normal adrenal glands, and 15 metastatic carcinomas to the adrenal gland of various histologies (6 clear cell renal cell carcinomas, 1 papillary renal cell carcinoma, 1 renal cell carcinoma, unclassified, 1 small cell carcinoma, 1 Merkel cell carcinoma, 2 colorectal carcinomas, 1 pancreatic adenocarcinoma, 1 cholangiocarcinoma, and 1 squamous cell carcinoma) were selected from our database. Hematoxylin and eosin-stained slides were reviewed to choose the most appropriate formalin-fixed paraffin-embedded tissue blocks for immunohistochemistry (IHC). Sections of 3 μm thickness of each selected block were prepared, and these were deparaffinized and rehydrated before antigen retrieval.

Antigen retrieval was performed using FLEX Low pH Target Retrieval (Dako, Carpinteria, CA) in a PT Module (Dako) for 20 minutes. After cooling and rinsing, the slides were placed on the AutoStainer Plus (Dako) and stained using a mouse monoclonal Oct4 (Cell Marque, Rocklin, CA) ready-to-use antibody and FLEX+ detection kit (Dako). The slides were visualized with FLEX DAB and counterstained with FLEX hematoxylin.

In addition, 7 cases of pheochromocytoma and 2 cases of ACC were selected to undergo immunostaining with a separate OCT4 antibody. This antibody was a polyclonal goat anti-OCT4 antibody (C20, sc 8629; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution, 30 min at room temperature) similar to that used in prior studies[1,2,7,8,11] and is also directed toward the C-terminus. Antigen retrieval was carried out by heating sections in 1 mmol/L ethylene diamine tetraacetic acid (pH 8.0) for 30 minutes. Endogenous peroxidase activity was inactivated by incubation in 3% H_2O_2 for 15 minutes.

To ascertain that the immunoexpression is not the result of denaturing of the proteins during formalin fixation, processing, and paraffin embedding, we performed IHC staining on fresh-frozen tissue from 4 cases of pheochromocytoma by utilizing both monoclonal and polyclonal antibodies.

To further evaluate the specificity of the antigen-antibody reactivity seen, a blocking peptide study used in conjunction with the polyclonal antibody was utilized on these same 9 cases. Nonspecific binding sites were blocked using Protein Block (Dako Corporation) for 20 minutes to further define the specificity of the antigen-antibody reaction seen. This product utilized casein, a hydrophilic protein that reduces background staining and allows for easier recognition of true antigen-antibody interactions.

After immunostaining of the slides was completed, the slides were reviewed and scored on a 2-criterion system (intensity and extent of staining) with a 4-tier (0 to 3) grading schema. All positive areas were correlated with morphology to confirm staining of the area of interest and was further confirmed with hematoxylin and eosin, if necessary.

For ultrastructural studies, formalin-fixed tissue was embedded in Unicryl plastic resin. Thin sections (70 to 90 nm) were mounted on Formvar/carbon-coated nickel grids. Blocking agents were applied for 45 minutes before overnight incubation with an undiluted primary antibody at 4°C. The sections were rinsed in phosphate-buffered saline and floated on drops of an appropriate secondary antibody coupled with 10 nm gold particles for 2 hours at room temperature. After fixing and rinsing the grids, they were stained with uranyl acetate before viewing with an FEI Tecnai Bio Twin transmission electron microscope operated at 80 kV. Images were captured with an AMT XR 60 digital camera.

RESULTS

Sixty-four cases including 30 pheochromocytomas, 24 ACCs, and 10 normal adrenal glands were examined by OCT4 IHC utilizing monoclonal antibody from Cell Marque. In 30 of 30 (100%) cases of primary pheochromocytoma, strong and diffuse (3+3) immunoexpression was observed (Figs. 1A, B). In the cases of metastatic pheochromocytoma, all cases showed diffuse staining. Six of 8 (75%) metastatic pheochromocytomas showed strong expression (3+3), whereas the remaining 2 (25%) showed moderate intensity (2+3) (Figs. 1C, D). In all positive-staining cases, the staining pattern was uniformly cytoplasmic, and distinct nuclear staining was not identified. In many cases, the intense cytoplasmic immunostaining overshadowed the nuclei.

In 24 of 24 (100%) cases of ACC, including metastatic cases, OCT4 was completely negative (Figs. 1E, F). In the population of metastatic tumors to the adrenal gland, weak cytoplasmic reactivity was present in only 2 cases, the Merkel cell carcinoma and the small cell carcinoma cases (Figs. 2A, B). Weak, focal reactivity was seen in 2 of the cases of sarcomatoid clear cell renal cell carcinoma within the dedifferentiated component. In all other cases, no cytoplasmic expression was present.

As a control tissue type for the pheochromocytoma cases, 10 cases of normal adrenal gland to include medulla were also analyzed for OCT4 staining. In all 10 cases, the 3+3 staining was seen in the normal medullary tissue, with a cytoplasmic staining pattern identical to that seen in the cases of pheochromocytoma. There was no staining seen in the normal adrenal cortex.

Immunostaining with the polyclonal OCT4 from Santa Cruz demonstrated the same cytoplasmic staining pattern as seen with the primary monoclonal antibody used in 6 of the 7 cases of pheochromocytoma evaluated (Figs. 2C, D). Only a slight blush was seen in the remaining case, and this was scored as negative. Though the pattern was the same, the intensity of staining was much less than that seen with the primary OCT4 monoclonal antibody. All positive cases were either scored at 1+ or 2+ intensity. Use of a blocking peptide on these cases demonstrated a complete loss of immunoreactivity or dramatic reduction in the staining pattern visualized (Fig. 2E). No reactivity was seen with this antibody in the 2 cases of ACC examined. All 4 cases of fresh-frozen tissue from pheochromocytomas showed positive immunostaining with both antibodies, intense cytoplasmic expression with the monoclonal antibody, and weak

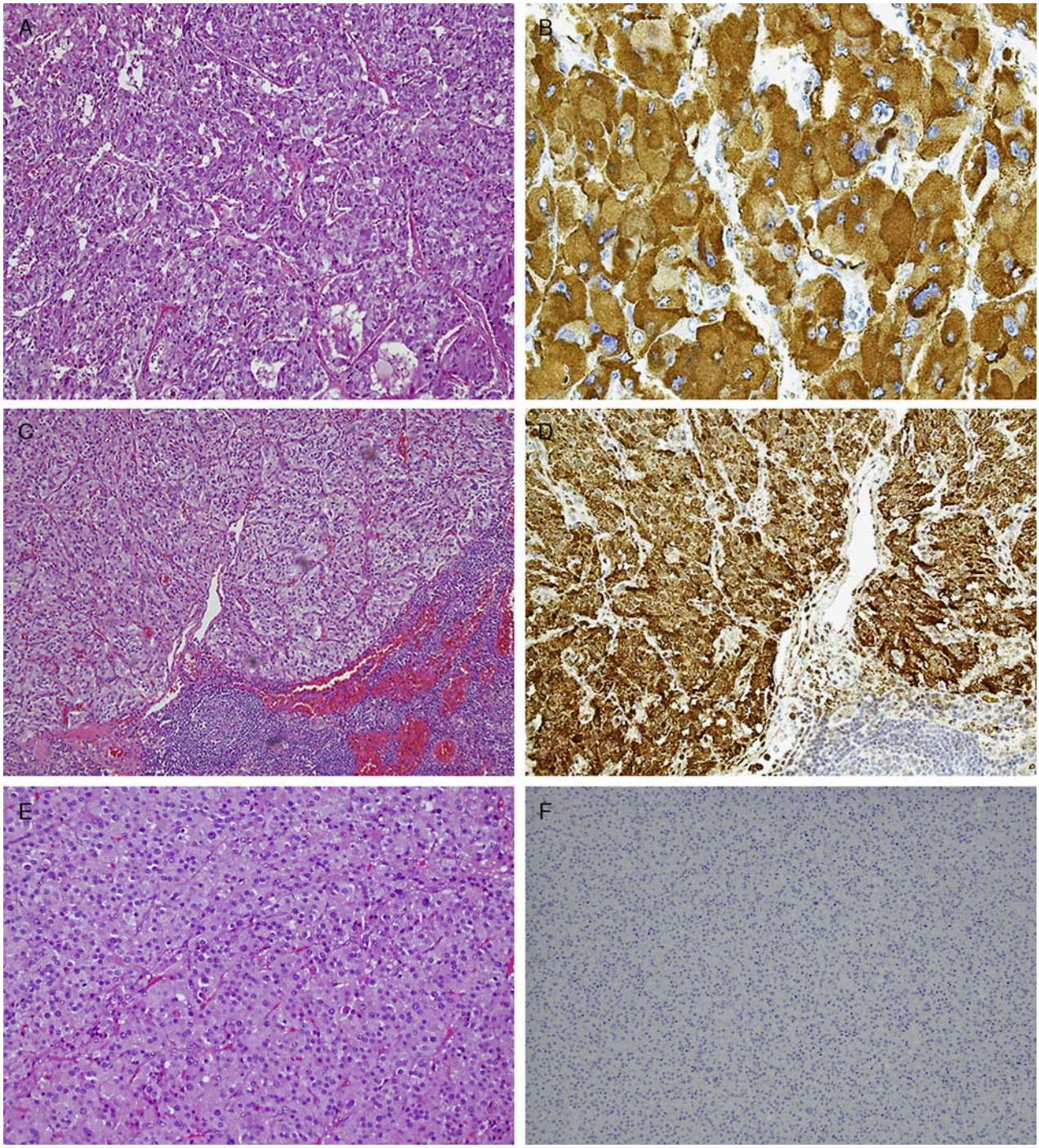

FIGURE 1. A, Hematoxylin and eosin-stained image of primary pheochromocytoma. B, High-power view of OCT4 (OCT4; Cell Marque, Rocklin, CA) immunostaining in the same case demonstrating strong cytoplasmic immunoreactivity with lack of nuclear immunoreactivity. C, Metastatic pheochromocytoma (hematoxylin and eosin) with adjacent benign lymphoid tissue. D, Strong, cytoplasmic OCT4 (OCT4, Cell Marque) immunoreactivity is seen in the metastatic pheochromocytoma with no staining seen in the adjacent normal tissue. E, Hematoxylin and eosin-stained image of adrenal cortical carcinoma. F, OCT4 (OCT4, Cell Marque) immunoreactivity is completely absent in adrenal cortical carcinoma.

cytoplasmic expression with the polyclonal antibody. This provided further evidence that the immunostaining is not the result of denaturing of proteins during formalin fixation, processing, and paraffin embedding.

Ultrastructural studies with immunoelectron microscopy were successful in locating the gold-tagged OCT4 monoclonal antibody, which was performed on normal adrenal medullary tissue and tissue from

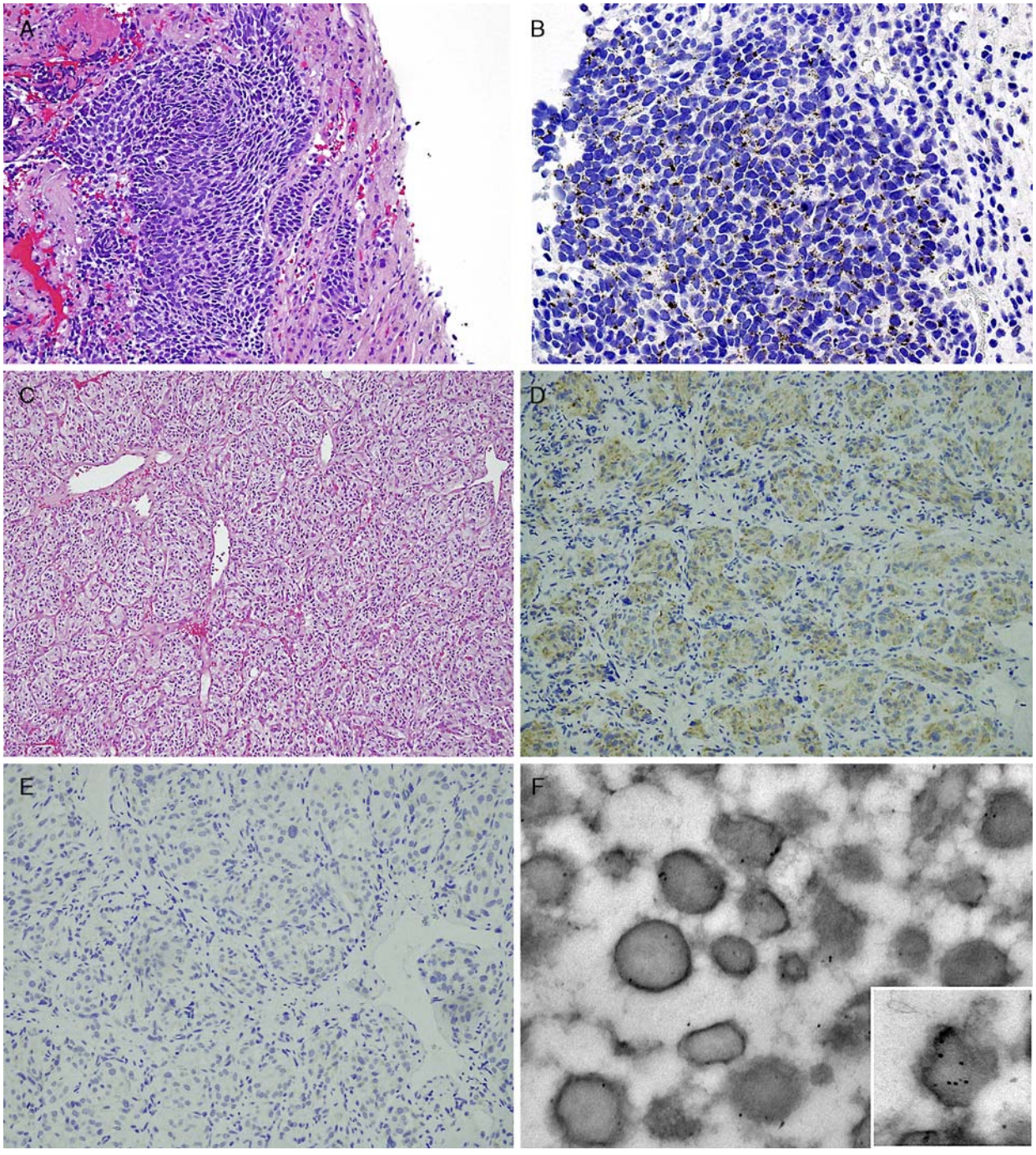

FIGURE 2. A, Metastatic small cell carcinoma in the adrenal gland (hematoxylin and eosin). B, Small cell carcinoma shows weak focal cytoplasmic positivity, likely correlating to scant cytoplasm and loss of neuroendocrine granules. C, Pheochromocytoma with classic architecture (hematoxylin and eosin). D, Immunostaining with the polyclonal OCT4 antibody used in many previous studies (C20, sc 8629; Santa Cruz Biotechnology) shows the same pattern of staining with less intensity. E, Treatment with a blocking peptide directed toward the OCT4 antigen eliminates nearly all immunoreactivity and demonstrates that the immunoreactivity is not nonspecific background staining. F, Immunoelectron microscopy of normal adrenal medulla showing discrete reactivity to the gold-tagged OCT4 antibody within the cytoplasm. These structures correspond to neurosecretory granules. The inset highlights a brisk reactivity within the neurosecretory granules.

pheochromocytoma. Visualization of the obtained images clearly showed the antibodies adhering to the neurosecretory granule matrix within the cytoplasm (Fig. 2F). Background staining was minimal to nonexistent. Control slides of seminoma demonstrated the characteristic and classic nuclear staining for OCT4.

DISCUSSION

Our findings show that the cytoplasmic OCT4 staining pattern observed is a sensitive marker of both pheochromocytoma and normal adrenal medulla, especially when utilizing a monoclonal OCT4 antibody. From our results, it appears that there is staining homogeneity among cases of obvious malignant pheochromocytoma (metastatic cases) and in those that may be termed benign, in addition to the physiologically normal adrenal medulla. Previous reports identifying cases of OCT4 positivity in germ cell neoplasia utilizing polyclonal antibodies only considered nuclear staining as a positive, yet no comments were made toward the cytoplasmic staining described in our results.[1,2,6,8]

In the large study by Looijenga and colleagues, 26 cases of pheochromocytoma were analyzed with OCT4 and demonstrated no reactivity in all cases. They also stained and analyzed 10 cases of paraganglioma and 19 cases of ACC, neither group displaying any reported immunoreactivity.[8] Cheng's[11] examination of multiple non–germ cell neoplasms did not assess any adrenal malignancies. It should be taken into account that all the prior studies were conducted using the polyclonal OCT4 antibodies. Therefore, to our knowledge, this paper is the first to characterize this particular OCT4 staining pattern in pheochromocytoma utilizing the polyclonal and monoclonal antibodies.

The early work examining the sensitivity and specificity was thorough and broad reaching. The aforementioned study by Looijenga et al[8] examined over 100 tissue types in over 3600 cases and only found positivity in 4 tumor types: clear cell renal cell cancer, squamous cell lung carcinoma, large cell lung carcinoma, and germ cell tumors. In germ cell tumors, all seminomas were positive, and 33 of 47 (70.2%) of mixed cases were positive. In the remaining tissue types, only 1 positive case of each tumor type was found, with a minimum of 47 cases per type.[8] A large study by Cheng[11] examining 84 non–germ cell metastases, however, failed to demonstrate any OCT4 positivity. In addition, a study that comprised 115 specimens, focusing on cutaneous neoplasia, failed to demonstrate any OCT4 reactivity in multiple neoplastic entities.[16] With the work by Jones et al[1,2] further subclassifying the germ cell specificity to seminoma, embryonal carcinoma, and intratubular germ cell neoplasia, it was felt that the marker was limited to these tissue types with a very high degree of specificity.

In all of these cases, the staining pattern defined was nuclear. In addition, all of the works published at our institution and that by Looijenga and colleagues used the same antibody, the polyclonal goat anti-OCT4 antibody (C20, sc 8629; Santa Cruz Biotechnology; 1:500 dilution, 30 min at room temperature) directed toward the COOH terminus of the OCT4 protein.[1,2,7,8,11]

Zuk[5] conducted a comprehensive study on multiple OCT4 antibodies to elucidate differences in staining patterns among antibodies in ESCs. Interestingly, a wide array of markers was studied, and many of the results were unexpected and inconsistent with current theories on isomer distribution. Polyclonal and monoclonal antibodies directed at OCT4A/B failed to express a nuclear component in many cases. Even monoclonal antibodies directed toward the N-terminus of OCT4A, which is specific for the nuclear-localized isomer, failed to show nuclear staining in some cases.[5] Unfortunately, the polyclonal goat antibody from Santa Cruz and the monoclonal antibody from Cell Marque used in this study were not investigated.

Although the results from Zuk's work leave a doubt regarding the reproducibility of different antibodies used for OCT4 immunoreactivity, much recent work has been conducted that may explain some of this variation. The cancer stem cell has grown to have a significant role in hypothesizing the tumorigenic, metastatic, and recurrence patterns of cancer.[17] OCT4, as a marker of pluripotency, has become a surrogate marker for these cells.[5,7,12,13,18] Investigations into their roles have revealed the presence of OCT4 in colorectal cancer, lung adenocarcinoma, gliomas, and gastric cancers in humans.[4,12–15] Animal studies have even shown the presence of characteristic OCT4 staining, of a cytoplasmic nature, in pancreatic cancer models.[19] Although a detailed look at all of these entities is beyond the scope of this paper, they have resulted in the discovery of additional OCT4 variants that may help explain some of the variability reported with different OCT4 antibodies.

To address this issue, we performed immunostaining with this same antibody, the polyclonal goat anti-OCT4 antibody (C20, sc 8629; Santa Cruz Biotechnology; 1:500 dilution, 30 min at room temperature) directed toward the COOH terminus of the OCT4 protein in 7 of our cases of metastatic pheochromocytoma, to determine whether the staining pattern described by us was also seen with this antibody. In 6 of the 7 cases, the same cytoplasmic staining pattern was present, although with less intensity. It is likely that either the amount of OCT4 antigen-binding sites in the cytoplasm of these cases is lower than that seen in germ cell tumors, that the polyclonal antibody has a lower affinity for the antigen binging site, or that there is some combination of the 2. Determination of this is outside the scope of this work; however, both antibodies displayed the described cytoplasmic staining pattern, and this weakens the argument that this particular reactivity is limited to a single antibody. In addition, the weaker intensity of the polyclonal antibody used in many of the prior studies tempers the question as to why this staining was not previously described.

The variability of both antibody and antigen may explain some of the differences in expression patterns seen in previous studies; however, our results indicate that this variability is not the reason for our findings. By conducting immunoelectron microscopy, we have shown that the antibody used in our study interacts directly with neurosecretory granules. This would imply that localization of OCT4 to neurosecretory granules occurs in these cell types or that the granules express an epitope that reacts to the antibody by antigenic mimicry. The

significant decrease or total loss of OCT4 reactivity in the presence of a blocking peptide also supports the assertion that this staining is true OCT4 immunoreactivity and not background staining.

The electron microscopic confirmation of antibody binding to neurosecretory granules suggests that similar immunoreactivity to OCT4 may be seen in other neuroendocrine tissues and tumors. The only tumors of neuroendocrine differentiation examined in the large series by Looijenga et al[8] and Cheng[11] were small cell and large cell carcinomas—only 1 case of large cell carcinoma was positive in the studied population from both studies. Our report demonstrated cytoplasmic staining in the single cases of Merkel cell and small cell carcinomas examined. This staining was less intense than that seen in the adrenal medulla or pheochromocytoma, but the granular pattern seen may be attributed to residual neurosecretory granules within these high-grade neuroendocrine tumors with little cytoplasm.

Further reports in the literature of OCT4 expression in neuroendocrine tissues are sparse. Sotomayor et al[20] describe OCT4 immunoreactivity in a subset of cells within the prostate that were determined to be neuroendocrine cells by cross-reactivity with synaptophysin and chromogranin-A. Interestingly, they reported a strong degree of cytoplasmic expression with concurrent nuclear expression. An additional step using a blocking peptide specific for OCT4A was utilized, as in our study, and confirmed that the reactivity seen in both nucleus and cytoplasm was due to the presence of OCT4A.[20]

The particular monoclonal OCT4 antibody utilized in our study differs from that used in other studies examining immunoreactivity in multiple tissue types. The most obvious dissimilarity is that the antibody used in our study is monoclonal as opposed to the polyclonal goat antibody used in the studies referenced in this report.[1,2,7,8,11] One possible explanation for this difference could be attributed to possible epitope variation between the 2. However, both the Cell Marque antibody used in our study and the Santa Cruz polyclonal goat antibody used in the previously referenced studies displayed reactivity at the carboxyl-terminus of OCT4, which is identical in OCT4A and OCT4B.[7] Therefore, this explanation does not explain the intense cytoplasmic staining seen in our cases of pheochromocytoma and the lack of it reported previously. Higher affinity and specificity of monoclonal antibody may attribute to the intense immunostaining observed in our study.

CONCLUSIONS

We have shown that cytoplasmic staining of OCT4 is a highly sensitive marker of the adrenal medulla and its related neoplastic tissue. The strong reactivity and high sensitivity of this antibody in this setting suggests that it may be a useful diagnostic adjunct when it is necessary to distinguish between a cortical and a medullary lesion. Although both monoclonal and polyclonal antibodies display similar cytoplasmic staining, the stronger expression by the monoclonal antibody makes it more attractive to be used in diagnostic studies.

By using immunoelectron microscopic methods, we have further shown that the neurosecretory granules within these cells are the target of the antibody. Comparison studies with the polyclonal Santa Cruz OCT4 antibody used in many of the early publications on OCT4 demonstrate that this pattern is not limited to the Cell Marque antibody utilized in this study, despite the weaker intensity seen. In addition, the utilization of the blocking peptide studies confirms that the antigen site within the neurosecretory granules is OCT4 and is not nonspecific background staining.

Considering these findings, the weak reactivity seen in our examined cases of small cell carcinoma and Merkel cell carcinoma and the limited evidence in the literature, the authors hypothesize that the cytoplasmic staining pattern reported may not be specific to adrenal medullary tissue but may likely be a general marker of neuroendocrine differentiation. A follow-up study is currently underway investigating multiple neuroendocrine tumors to determine the validity of this assertion.

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