Original Paper

Nuclear CK19-immunopositive pseudoinclusions as a new additional objective diagnostic feature of papillary thyroid carcinoma

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One of the key parameters in the diagnosis of papillary thyroid carcinoma (PTC) are true nuclear pseudoinclusions (NPs), which constitute invaginations of the cytoplasm into the nucleus. On the other hand, strong cytoplasmic expression of CK19 is a well-known attribute of PTC tumor cells. We analyzed NPs using CK19 immunohistochemistry in histological sections of 52 PTCs and seven noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTPs). Strong CK19+ NPs were present in 77% of PTCs, whereas NPs in hematoxylin and eosin (HE)-stained slides (HE NPs) were identified in only 48% of PTCs. Detection of CK19+ NPs enabled easier and objective recognition of NPs and better discrimination of NPs from pseudo-pseudoinclusions than detection of HE NPs. In the 15 of the 27 (55.5%) PTCs in which we could not discern HE NPs, strong CK19+ NPs could be identified reliably, quickly and easily. Moreover, all NIFTPs were negative for both CK19+ NPs and HE NPs. Detection of CK19+ NPs may refine the assessment of this important diagnostic feature and, hence, the microscopic diagnostic criteria of PTC. Thus, these findings may have implications for the accurate diagnosis of PTC and NIFTP.

Key words: nuclear pseudoinclusions, papillary thyroid carcinoma, CK19.

Introduction

It is generally agreed that no single cyto-architectural feature is diagnostic of papillary thyroid carcinoma (PTC). Instead, diagnosis of PTC requires the presence of a set of morphological nuclear features including (a) changes of nuclear size and shape, (b) nuclear membrane irregularities, including true nuclear pseudoinclusions (NPs), and (c) distinctive chromatin attributes [1]. Since nuclear characteristics, especially NPs, are key parameters in the diagnosis of PTC, their objective assessment is of utmost importance. In hematoxylin and eosin (HE)-stained histological sections, NPs (HE NPs), which constitute intranuclear cytoplasmic invaginations, are seen as acidophilic round structures delimited by the nuclear membrane [2]. Electron microscopic studies have shown that NPs contain various cytoplasmic organelles [3]. NPs have been reported as one of the five most diagnostic features of PTC (in addition to a syncytial-type arrangement; pale, enlarged nuclei; nuclear grooves; and multiple nuclei) [4]. Stepwise logistic regression analysis also indicated that NPs are one of the important predictive diagnostic features of PTC [5]. In addition, NPs have been suggested as a discriminative parameter between noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and PTC [6].

Thus, NPs, although not totally specific, are important diagnostic nuclear features of PTC. Nuclear pseudoinclusions are regarded as major and helpful diagnostic feature for all variants of PTC, although they are uncommon in encapsulated follicular variant

of PTC [7]. NPs have been reported in various percentages of HE-stained PTCs [8, 9]. However, distinguishing HE NPs from pseudo-pseudoinclusions (i.e., "hollow", pale, poorly demarcated artifactual bubbles in nuclei; [2]) on histological slides is often difficult and error-prone because this approach is based on subjective assessment. Some pseudo-pseudoinclusions may be mistaken for NPs, and *vice versa*. Moreover, pseudo-pseudoinclusions that mimic NPs or clear nuclei can sometimes lead to misdiagnosis of follicular adenoma or hyperplastic nodule as PTC. Therefore, objective methods for assessing NPs that could more accurately distinguish them from pseudo-pseudoinclusions are needed.

Strong and diffuse cytoplasmic expression of CK19 has been repeatedly reported in PTC tumor cells [10, 11, 12, 13, 14, 15, 16]; thus, we hypothesized that NPs might exhibit CK19 expression because they constitute invagination of the cytoplasm into the nucleus. Therefore, we analyzed NPs using CK19 immunohistochemistry, aiming to refine the assessment of this important diagnostic feature and, hence, the microscopic diagnostic criteria for PTC. Here, we report strong dot-like expression of CK19 in NPs (CK19+ NPs) as an additional objective diagnostic feature of PTC.

Material and methods

This study included 52 unselected PTCs and 7 NIFTPs diagnosed between 2018 and 2019. Among the PTCs 40 were papillary microcarcinomas (≤ 10 mm in diameter) and 12 were > 10 mm in diameter. All available HE-stained histological slides routinely fixed in buffered 10% formalin and the corresponding slides immunostained for CK19 (using a monoclonal CK19 antibody (clone RCK108) and a Dako EnVision™ kit) were retrieved for each patient from the archives of the Department of Pathology, Pomeranian Medical University in Szczecin, Poland. The slides were reexamined independently by two pathologists (PD and WD) according to published diagnostic criteria for PTC [1] and NIFTP [17] and representative slides were selected for further analysis. In total 46 classic papillary carcinomas, three follicular variants and three oxyphilic variants were identified. Two histological slides for each tumor were assessed: an HE-stained section and a section immunostained for CK19.

The number of HE NPs and the number of NPs exhibiting strong dot-like expression of CK19 (CK19+ NPs) in the corresponding section immunostained for CK19 were counted independently by the two pathologists, and the mean of both counts was calculated. According to the number of NPs per slide, (0, 1-4, 5-10, and > 10 NPs on a slide) the PTCs were assigned to four groups. Furthermore, the numbers and percentage of tumors exhibiting various combinations of HE and CK19+ NPs (HE+ & CK19+; HE– & CK19–; HE– & CK19+; HE+ & CK19–) were calculated. Fisher's exact test was used for categorical variables to determine differences between groups, a p value < 0.05 was considered significant.

Results

In the HE-stained histological sections, HE NPs, which constitute intranuclear cytoplasmic invaginations, were seen as acidophilic, round or oval, nuclear membrane-delimited structures of various sizes (Fig. 1). Occasionally, two HE NPs were identified in the same nucleus (Fig. 2). HE NPs differed from nuclear pseudopseudoinclusions and from nucleoli in that the latter were not surrounded by the nuclear membrane.

CK19 expression was visible as strong brown staining in the cytoplasm of tumor cells of all papillary carcinomas and microcarcinomas (except for

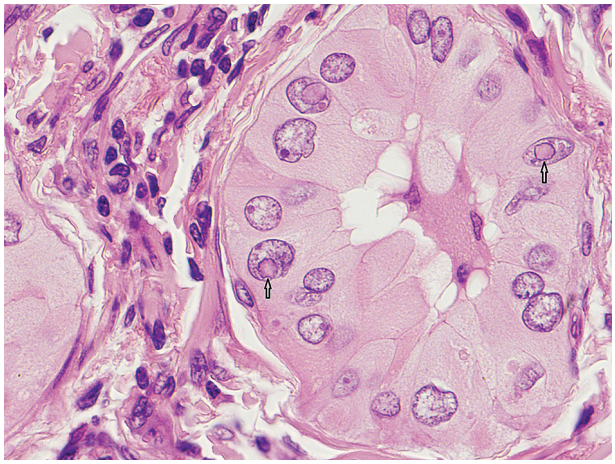

Fig. 1. Acidophilic, round, nuclear membrane-delimited NPs (arrows) in HE-stained histological section

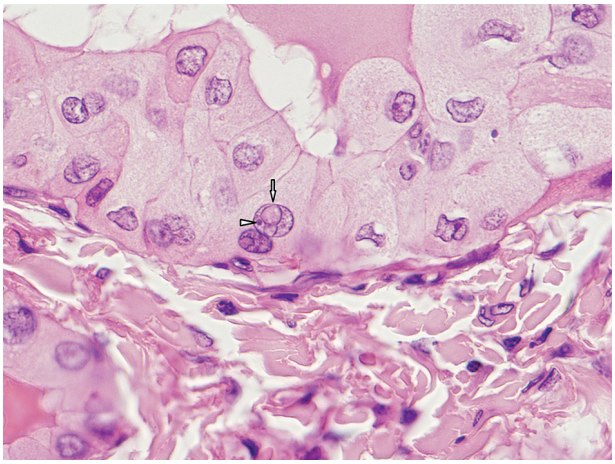

Fig. 2. Two NPs (arrow) and nucleolus (arrowhead) in the same nucleus. HE-stained histological section

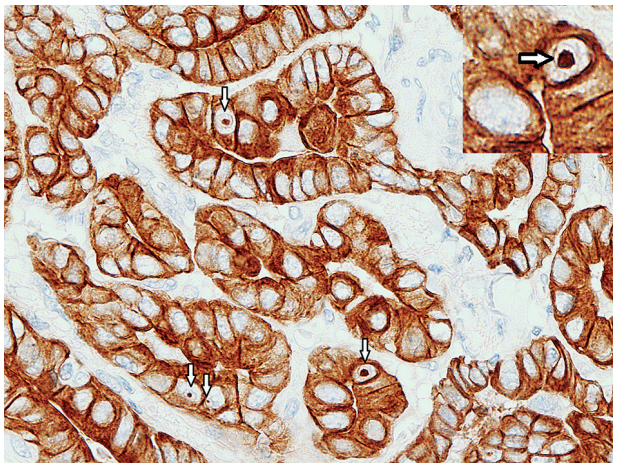

Fig. 3. CK19+ NPs of various sizes (arrows). Inset: high power image of one tumor cell (arrow) exhibiting a large NP with strong CK19 immunoreactivity and another nucleus without NP (lower left corner). Note the nuclear membrane surrounding the CK19+ NP. CK19 expression is also observed in the cytoplasm of the tumor cell. Immunohistochemistry was performed with an anti-CK19 monoclonal antibody

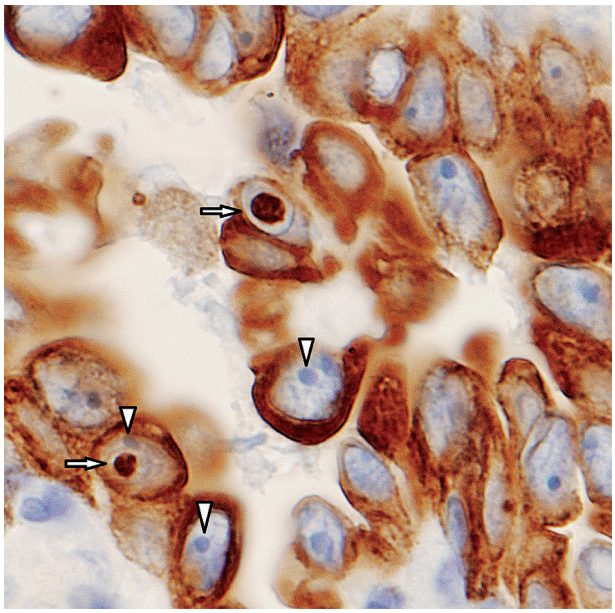

Fig. 4. Two brown CK19+ NPs (arrows). Note the difference between CK19+ NPs (brown) and nucleoli (blue), which are negative for CK19 (arrowheads). The nucleus on the left side contains CK19+ NP and the nucleolus. Immunohistochemistry was performed with an anti-CK19 monoclonal antibody

one microcarcinoma). Moreover, in some tumor cell nuclei, we identified round or oval, well-delineated NPs exhibiting strong dot-like expression of CK19 (CK19+ NPs) (Fig. 3). These pseudoinclusions were surrounded by the nuclear membrane (Fig. 3, inset) and differed from nuclear pseudopseudoinclusions and nucleoli (Fig. 4). Moreover, these CK19+ NPs were found more quickly and easily than HE NPs, the identification of which required more time and effort. Rarely, two CK19+ NPs could be seen in the same nucleus (Fig. 5). CK19+ NPs were variably sized, from very small (pinpoint-sized) to very large, much larger than the average nucleolus (Fig. 3).

In total, nearly 77% of PTCs were positive for CK19+ NPs, whereas HE NPs were found in only 48% of PTCs ($p = 0.004$, Table I). In almost half of all PTCs (48%), both HE NPs and CK19+ NPs were found. In contrast, in 28.8% of the PTCs, CK19+ NPs but no HE NPs were found (Table II).

CK19+ NPs were found in 28/40 (70%) microcarcinomas and 12/12 (100%) PTCs > 10 mm ($p = 0.04$) whereas HE NPs were found in only 17/40 (42.5%) microcarcinomas and 8/12 (66.6%) PTCs > 10 mm ($p = 0.19$). Altogether, 17/40 microcarcinomas (42.5%) exhibited both HE NPs and CK19+ NPs, and 11/40 (27.5%) microcarcinomas were negative for HE NPs but positive for CK19+ NPs (Table II). A total of 12 microcarcinomas were negative for both HE NPs & CK19+ NPs; however, among the PTCs > 10mm no tumors were negative for both types of NPs (HE NP– & CK19+ NP–; Table II).

Of the 4 microcarcinomas with a diameter of 1 mm, 3 cases were negative for both HE and CK19+ NPs and one was negative for HE NPs but positive for CK19+ NPs. In one microcarcinoma (9 mm in diameter), no HE NPs were detected, but more than 10 CK19+ NPs were seen.

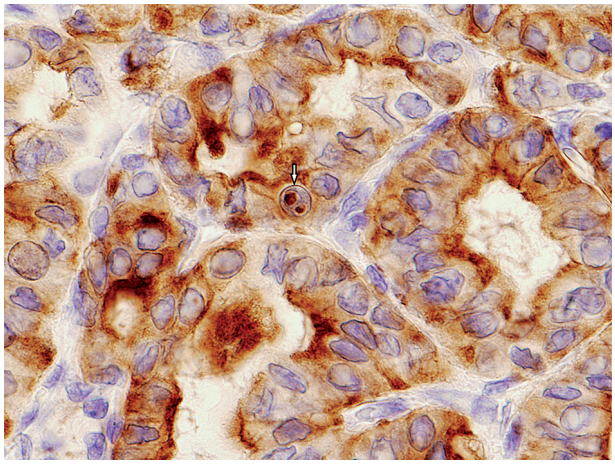

Fig. 5. Two CK19+ NPs in the same nucleus (arrow). Immunohistochemistry was performed with an anti-CK19 monoclonal antibody

Of the 27 PTCs (23 microcarcinomas and 4 PTCs > 10 mm in diameter) with no HE NPs, CK19+ NPs were found in 15 cases (55.5%). Of the 23 HE NP-negative microcarcinomas, 11 were positive for CK19+ NPs (47.8%). Of the 12 PTCs > 10 mm in diameter, only 4 were negative for HE NPs, but CK19+ NPs were discernible in these four tumors (Table II).

Table I. Number (%) of tumors with HE NPs or CK19+ NPs

NPs	Number of NPs per histological section				
	0	1-4	5-10	> 10	> 0
HE	27 (51.9)	11 (21.1)	8 (15.3)	6 (11.5)	**25 (48.1)**
CK19+	12 (23.1)	9 (17.3)	12 (23.1)	19 (36.5)	**40 (76.9)**

NPs – nuclear pseudoinclusions; HE NPs – nuclear pseudoinclusions in hematoxylin and eosin stained sections; CK19+ NPs – nuclear pseudoinclusions CK19 positive

Table II. Tumors grouped according to the number of PTCs with HE NPs and CK19+ NPs

NPs	All	Microca	PTC > 10mm
HE NPs (+) & CK19+ NPs (+)	25/52 (48.1%)	17/40 (42.5%)	8/12
HE NPs (–) & CK19+ NPs (–)	12/52 (23.1%)	12/40 (30.0%)	0/12
HE NPs (–) & CK19+ NPs (+)	15/52 (28.8%)	11/40 (27.5%)	4/12
HE NPs (+) & CK19+ NPs (–)	0/52	0/40	0/12

PTC – papillary thyroid carcinoma; Microca – microcarcinoma; NPs – nuclear pseudoinclusions; HE NPs – nuclear pseudoinclusions in hematoxylin and eosin stained sections; CK19+ NPs – nuclear pseudoinclusions CK19 positive

Of the 25 PTCs with HE NPs and CK19+ NPs, the number of CK19+ NPs was higher than that of HE NPs in 14/25 PTCs (56%), equal in 10/25 (40%) and lower in one case (exhibiting weak CK19 staining).

In summary, in the 55.5% of the 27 PTCs in which we could not discern HE NPs, strong dot-like CK19+ NPs could be found reliably, quickly and easily. Moreover, all NIFTPs were negative for both CK19+ NPs and HE NPs.

Discussion

In this report, we document NPs exhibiting strong dot-like expression of CK19 (dot-like CK19+ NPs) as one of characteristics of PTC nuclei. To our knowledge, this report is the first to describe this new additional objective feature of PTC. Since NPs are considered one of the most unequivocal diagnostic features of PTC, our results suggest that dot-like CK19+ NPs can help to refine the assessment of this feature and, hence, the microscopic diagnostic criteria for PTC. Here we report CK19+ NPs in histological sections of PTCs but one can envision their presence also in fine-needle aspirates of PTCs processed as cell blocks. For obvious reasons one should not expect CK19+ NPs to appear in CK19 negative PTCs. Nucleolar expression of CK19 has been suggested to be found in thyroid papillary carcinoma [18]; however, we believe that the authors mistook CK19 expression in NPs for nucleolar expression of CK19 because CK19+ dots they showed were membrane bound whereas the nucleoli are not delimited by the membrane.

There are two morphologically distinct types of nuclear inclusions with different diagnostic significance: true nuclear inclusions (e.g., accumulation of viruses in nuclei) and NPs. In HE-stained sections of PTCs, NPs are seen within the nucleus as acidophilic, round structures delimited by the nuclear membrane. In addition to light microscopy, ancillary techniques such as electron microscopy and confocal microscopy [19] have been used to visualize NPs. Ultrastructural studies have shown that various cytoplasmic organelles constitute the content of NPs [3].

There are two major problems associated with NPs. First, recognizing NPs may be difficult because overstaining of nuclei by hematoxylin may lead to difficulties in the observation of nuclear details and might affect the detection rate of nuclear grooves and NPs [20]. Second, misinterpretation of NPs can lead to diagnostic errors. For example, differentiation between NPs and nuclear pseudo-pseudoinclusions ("empty" nuclear vacuoles) may be difficult and lead to the misdiagnosis of follicular adenoma or hyperplastic nodule as PTC.

Our results indicate that dot-like CK19+ NPs enable straightforward recognition of NPs and improved differentiation of NPs from pseudo-pseudoinclusions because the latter do not contain cytoplasmic elements and are thus CK19-negative, "empty" nuclear vacuoles. Furthermore, various nuclear features mimicking those of PTC can be found in benign thyroid lesions; thus, the lack of NPs as evaluated by this objective method (dot-like CK19+ NPs) may sometimes help to overcome diagnostic difficulty and suggest against PTC.

Nuclear pseudoinclusions in papillary thyroid carcinoma and other thyroid lesions

Nuclear pseudoinclusions are particularly common in PTC. We found strong dot-like CK19+ NPs in 77% of PTCs, whereas NPs in HE-stained sections of the same PTCs were present in only 48%. In HE-stained sections of classical PTC, NPs were reported

in various percentages of cases, from 80-85% [8] to 100% (25/25 PTCs) [9]. Nuclear pseudoinclusions are found at a relatively low frequency in the follicular variant but are prominent in the tall cell variant of PTC [21]. Occasional NPs were reported in a case of medullary thyroid carcinoma [22], two cases of follicular tumors and one case of Hürthle cell carcinoma [23]. Nuclear pseudoinclusions are frequently seen in hyalinizing trabecular adenoma [24]. However, these estimates were based on analyses of HE-stained slides without objective supporting evidence. NPs are regarded as helpful diagnostic feature for PTC [1, 7] thus their presence based on an objective assessment (dot-like CK19+ NPs) together with other nuclear features strongly favors the diagnosis of PTC.

Noninvasive follicular thyroid neoplasms with papillary-like nuclear features, nuclear pseudoinclusions and nuclear pseudo-pseudoinclusions

The majority of NIFTPs exhibit a score NS2 and not NS3. A score of NS3 does not exclude NIFTP, but this nuclear score is seldom seen without true papillae (a lack of true papillae is a prerequisite for the diagnosis of NIFTP). Although the presence of NPs is accepted in the diagnosis of NIFTP [17], the number of NPs that may be present is unclear. The presence of NPs has been suggested to favor a diagnosis of PTC over a diagnosis of NIFTP [6, 25], and the presence of a large number of NPs should prompt careful investigation in the direction of PTC [26]. However, because no objective methods for NP assessment are known, the exact frequency of NPs in NIFTP is not known. It has been suggested that NPs are usually absent or can be found very rarely in NIFTP [26]. Bizzarro *et al.* [6] compared the morphological features of NIFTPs and PTCs (using liquid-based cytology) and found NPs as differentiating parameters between NIFTP and PTC ($p < 00001$) although the number of cases they examined was low (6 NIFTPs and 38 PTCs). Howitt *et al*. [25] found NPs in fine-needle aspirates of 79% of classical PTCs and 0% of NIFTPs and in two recent reports based on a total of 67 cases of NIFTP, no NP was found [9, 27].

One reason for these ambiguities may be that the detection of NPs in HE-stained sections is prone to a lack of interobserver reproducibility because of the subjective mode of assessment. Moreover, inadequate fixation or overheating during routine tissue processing can produce nuclear pseudo-pseudoinclusions which may be misinterpreted as NPs. Nuclear pseudo-pseudoinclusions are observed quite often in NIFTP (37% in [9]), and in such instances, dot-like, CK19+ NPs seem to be a valuable objective tool for distinguishing pseudo-pseudoinclusions from NPs. In our study CK19+ NPs were present only in PTCs and were absent in NIFTPs. However, the number of cases of NIFTP in our study was low (similar to the report by Bizzarro *et al*. [6]). We believe that searching for dot-like CK19+ NPs can help to settle the unresolved issue of possible presence of NPs in NIFTPs; however, this issue requires further study.

Emerin [20] and ubiquitin [9] have been suggested for use in the differential diagnosis between NPs and nuclear pseudo-pseudoinclusions (artifactual "bubbles"). However, our approach offers an advantage over the use of these markers in that no additional immunohistochemical staining is needed beyond CK19 staining which is routinely used to confirm the diagnosis of PTC.

In conclusion, we report CK19+ NPs as a new additional objective diagnostic feature of PTC. In this study, these NPs were present only in PTCs and were absent in NIFTPs. CK19+ NPs facilitate the objective and reliable visualization, identification and counting of NPs. Assessment of CK19+ NPs is easy, quick and objective. Because NPs are considered one of the most unequivocal diagnostic features of PTC, our results suggest that CK19+ NPs can help to refine the assessment of this feature and, hence, the microscopic diagnostic criteria of PTC. Because these NPs are objective characteristics, they can be regarded as a reliable additional supportive microscopic feature of PTCs. The findings may have implications for the accurate diagnosis of PTC and NIFTP.

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