ORIGINAL ARTICLE

Morules and morule-like features associated with carcinomas in various organs: report with immunohistochemical and molecular studies

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Background: Morules have been reported in pulmonary blastoma (PB), well differentiated fetal adenocarcinoma of the lung (W DFA), and uterine endometrioid carcinoma (EC), and rarely in other carcinomas. β Catenin gene mutation has been associated with morule formation.

Aims: To compare and clarify the cellular characteristics of morules in carcinomas in various organs and show that morules are distinct from epithelial cellular nodules.

Methods: Twenty tumours were studied: two PBs, three W DFAs, three papillary lung adenocarcinomas, 11 ECs, and one papillary thyroid carcinoma. Numerous epithelial cell, oncofetal, and neuropeptide antibodies were used for immunohistochemistry. β Catenin gene mutation was investigated.

Results: Morules in PBs and ECs were uniform cell clusters distinct from squamous differentiation. All were immunonegative for epithelial cell and oncofetal antigens, but those in ECs were positive for neurone specific enolase γ (NSE γ). Synaptophysin, encephalin, and somatostatin were sporadically immunopositive in PB morules. Morules were not seen in the other carcinomas and W DFAs, although morule-like features closely resembling morules histopathologically were seen. These were positive for epithelial cell and oncofetal antigens, and showed squamous differentiation. Their nuclei were more atypical and slightly larger than those in morules. Morule-like features were seen in W DFAs. β Catenin gene mutation was demonstrated in one EC and PB, and in two W DFAs.

Conclusion: Morules were non-epithelial cell clusters showing neuronal differentiation. There were two types: endometrioid type, expressing NSE γ , and blastoma type, expressing neuropeptides. In contrast, similar morule-like features were epithelial nodules. Although the number of cases was small, the presence of morules showed no clear prognostic correlations.

Morules have been reported in well differentiated fetal adenocarcinoma of the lung (W DFA) and in pulmonary blastoma (PB),^{1–6} and it has also been reported that morules show neuroendocrine differentiation. Morule formation in such tumours has also been reported to be associated with β catenin gene mutation.^{4–6} β Catenin is a submembranous component of the cadherin mediated cell to cell adhesion system. Previously, Sekine *et al* reported that β catenin gene mutation was found in PBs (two of two cases) and W DFAs (three of three cases).⁷ Saegusa and Okayasu⁶ reported that this gene mutation is present in 84.6% of uterine and ovarian endometrioid carcinomas with morules. Furthermore, Paracios and Gamallo⁸ reported mutation in five of 11 cases of ovarian endometrioid carcinoma, including one borderline tumour.

“The term squamous metaplasia is now used only in the case of benign lesions, whereas squamous differentiation is used for carcinoma and benign lesions”

In contrast, Fornelli and colleagues⁹ reported a rare case of bronchioloalveolar carcinoma with morule-like features, in which the morule-like nodules were composed of alveolar cells. Separately, morules have been seen in uterine endometrioid carcinoma (EC) and also in benign endometrial lesions, such as hyperplasia and chronic endometritis, and very rarely in thyroid carcinoma¹⁰ and colonic adenoma.¹¹ It is generally thought that they are peculiar squamous cell foci, categorised as relatively immature squamous metaplasia or squamous differentiation cell clusters.^{12–13} Morules consist of

uniform spindle or round cells, and show some optically clear nuclei.⁴

Formerly, the term squamous metaplasia was used both in adenocarcinoma cases and also in benign lesions, such as chronic inflammation and hyperplasia of the endometrium and other organs, but recently the term squamous differentiation has also become common.^{12–13} The term squamous metaplasia is now used only in the case of benign lesions, whereas squamous differentiation is used for carcinoma and benign lesions. In addition, it is known that squamous differentiation tissue in carcinomas is usually well differentiated and is associated with a relatively good prognosis.¹²

In our previous report,¹⁴ we showed that morules in uterine EC and also in benign endometrial lesions are completely different from squamous differentiation cell clusters: they did not react with antibodies to epithelial cell antigens, including involucrin, epithelial membrane antigen, and cytokeratins, but were positive for neurone specific enolase γ (NSE γ), and in a few cases somatostatin and acetylcholine esterase. Furthermore, neither oncofetal nor proliferative cell markers were demonstrated in the morules. Therefore, endometrial morules were thought to be neuroectodermal-like cell clusters or nodules. They were not infected with human papillomavirus, (HPV), whereas some of the true squamous differentiated tissue was associated with HPV infection. Detailed histological examination of the morules in various

Abbreviations: EC, endometrioid carcinoma; HPV, human papillomavirus; NSE γ , neurone specific enolase γ ; PB, pulmonary blastoma, PCR, polymerase chain reaction; W DFA, well differentiated fetal adenocarcinoma

Table 1 Antibodies used for immunohistochemistry

Antibody	Source	Mab or Poly
Epithelial markers		
Cytokeratin AE1/AE3	Dako, Glostrup, Denmark	Mab
Cytokeratin 5/6	Dako, Glostrup, Denmark	Mab
Cytokeratin 7	Dako, Carpinteria, California, USA	Mab
Cytokeratin 10	Dako, Glostrup, Denmark	Mab
Cytokeratin LP34	Dako, Glostrup, Denmark	Mab
Involucrin	Sigma, St Louis, Missouri, USA	Mab
EMA	Dako, Glostrup, Denmark	Mab
Nerve cell or neurosecretory products		
NSE γ	Dako, Glostrup, Denmark	Mab
S-100	Dako, Carpinteria, California, USA	Poly
Acetylcholine esterase	Chemicon International, Temecula, California, USA	Mab
GFAP	Invitrogen, Carlsbad, California, USA	Poly
Chromogranin A	Dako, Glostrup, Denmark	Mab
Synaptophysin	Dako, Glostrup, Denmark	Poly
Somatostatin	Dako, Carpinteria, California, USA	Poly
Enkephalin	Wako, Osaka, Japan	Poly
Oncofetal or proliferative cell markers		
CEA	Dako, Glostrup, Denmark	Mab
p53	Dako, Glostrup, Denmark	Mab
PCNA	Dako, Glostrup, Denmark	Mab
Ki-67	Dako, Glostrup, Denmark	Poly
Blood Group A	Dako, Carpinteria, California, USA	Mab
Blood Group B	Dako, Carpinteria, California, USA	Mab
Blood Group H	Dako, Carpinteria, California, USA	Mab
Others		
Apo-A	Dako, Kyoto, Japan	Mab
β Catenin	Santa Cruz Biotechnology, Santa Cruz, California, USA	Mab

CEA, carcinoembryonic antigen; EMA, epithelial membrane antigen; GFAP, glial fibrillary acidic protein; NSE γ , antibody (clone, BBS/NC/V1-H14) specific for human neurone specific enolase γ , no reaction with α and β ; Mab, monoclonal antibody; PCNA, proliferative cell nuclear antigen; Poly, polyclonal antibody.

organs is needed to clarify their biological characteristics. In this report, we determine the characteristics of morules in carcinomas in various tissues.

MATERIALS AND METHODS

Samples

Eleven samples of EC with morules were obtained from 106 cases of uterine EC treated in 2003 at the department of gynaecology and obstetrics, Ryukyu University Hospital, Japan. Three samples of papillary carcinoma of the lung with morule-like features (three of 577 cases; 0.52%, 69, 67, and 74 year old women), one sample each of PB (39 year old woman), and one sample of WDFA (31 year old woman) were from National Okinawa Hospital, Okinawa, Japan. They were drawn from 1592 cases of lung cancer, which included the 577 cases of papillary carcinoma, treated there in the period 1984–2004. One more case of PB (42 year old man; obtained 15 years before) was from Okinawa Prefectural Naha Hospital, Japan. One sample of thyroid carcinoma with morule-like features (one of 141 cases of papillary carcinoma; 0.71%) was obtained from 208 cases of thyroid carcinoma (141 papillary carcinomas, 65 follicular carcinomas, and two others) investigated in our laboratory in the same period 1984–2004. Two samples of WDFA with morules were from Toyama City Hospital, Toyama, Japan. Five samples each of squamous cell carcinoma and adenosquamous carcinoma of the lung from National Okinawa Hospital, Japan were used as controls. A further three cases each of ameloblastoma, neuroblastoma (both adrenal and olfactory cases), nephroblastoma, and medulloblastoma, and one case each of mixed germ cell tumour of the testis (14 year old boy) and yolk sac tumour of the ovary (20 year old woman) from Ryukyu University Hospital, Japan were examined.

Morphological examination

The tumours were fixed in 10% phosphate buffered formalin and routinely processed in paraffin wax. Haematoxylin and eosin, phosphotungstic acid haematoxylin, silver impregnation, and periodic acid Schiff staining were carried out on

4 μ m thick sections. For the immunohistochemical staining, 4 μ m thick dewaxed sections were pretreated with 3% H₂O₂ for 20 minutes, washed, and blocked with non-immune goat serum for 30 minutes. Table 1 lists the antibodies used. Immunohistochemistry was performed using a microwave oven heating antigen retrieval method. Briefly, sections were heated in 10mM sodium citrate buffer (pH 6.0) for three five minute cycles using a microwave oven. To clarify the histological characteristics of the morules, a variety of antibodies (antibodies related to epithelial cell antigens including squamous cell markers, such as cytokeratins, involucrin, and oncofetal protein; proliferative cell markers; and nerve cell and neuropeptide related antigens) were used.

After incubation with the primary antibodies, the sections were incubated with biotinylated secondary antibody with avidin and biotinylated horseradish peroxidase complex (Dako, Kyoto, Japan). 3,3'-Diaminobenzidine (Dako) was used as a chromogen. As negative controls, non-immunised sheep serum or distilled water was used instead of antibodies. Furthermore, biotin containing intranuclear inclusions were detected by peroxidase labelled avidin.

In situ hybridisation kits for HPV types 6, 11, 16, 18, 30, 31, 33, 35, 45, 51, and 52 (Dako, Carpinteria, California, USA) were used for the detection of HPV. In situ hybridisation was carried out according to the manufacturer's instructions.

Table 2 summarises the results of immunohistochemical investigations, including the results of controls.

Detection of β catenin gene mutation

DNA samples were routinely prepared according to the methods described by Imprim *et al.*¹⁵ Briefly, the morules were microdissected from 15 μ m dewaxed sections under the light microscope. After proteinase K (Merck, Tokyo, Japan) treatment, DNA was extracted using the phenol/chloroform method. The DNA was precipitated with three times the volume of 100% ethanol at -20°C . The β catenin gene was amplified by the polymerase chain reaction (PCR). The PCR primer set with sense 5'-ATGGAACCAGACAGAAAAGCG-3' and antisense 5'-CAGGATTGCCTTTACCACTCA-3' was used

Figure 1 (A) Morules in uterine endometrioid carcinoma. Immunostaining for epithelial membrane antigen (EMA). Staining for EMA is strong in the carcinoma cells (arrowheads), but not in the morules (asterisk). The carcinoma cells (arrowheads) were found to be pushed up by the morule into the glandular lumen and are seen on the margin of the morule. Original magnification, $\times 200$. (B) Morules in pulmonary blastoma. Arrowheads indicate the optically clear nuclei in the morule (asterisk). Haematoxylin and eosin (H&E) staining; original magnification, $\times 200$. (C) Biotin staining of pulmonary blastoma. Staining for biotin (arrowheads) was strong in optically clear nuclei. Original magnification, $\times 100$. (D) Well differentiated fetal adenocarcinoma of the lung. Immunostaining for EMA. Strong staining for EMA is seen in both morules (asterisk) and carcinoma cells (arrowheads). Original magnification, $\times 150$. (E) Papillary carcinoma of the thyroid. The asterisk indicates a morule-like feature. H&E staining; original magnification, $\times 150$. (F) Papillary adenocarcinoma of the lung. A morule-like feature (asterisk) is seen filling the lumen of the glandular structures of the carcinoma, but the nuclei are more atypical and slightly larger than those in true morules in the endometrioid carcinoma and pulmonary blastoma.

Table 2 Immunohistochemistry results

Antibody	Morules		Morule-like features			Controls	Squamous cell component in adenosquamous carcinoma
	A	B	C	D	E	SCC	
Cytokeratin AE ₁ /AE ₃	—	—	+	+	+	+	+
Cytokeratin 5/6	—	—	+	+	+	+	+
Cytokeratin 10	—	—	+	+	NT	+	+
Cytokeratin 14	—	—	+	+	NT	+	+
Cytokeratin LP34	—	—	+	+	+	+	+
Involucrin	—	—	+	+	+	+	+
EMA	—	—	+	+	+	+	+
NSE γ	—	+	—	—	—	—	—
S-100	—	—	—	—	—	—	—
Acetylcholine esterase	—	±	—	—	—	—	—
GFAP	—	—	—	—	—	—	—
Chromograin A	±	—	—	—	(+)	—	—
Synaptophysin	±	—	—	—	—	—	—
Somatostatin	±	—	—	—	(+)	—	—
Enkephalin	±	—	—	—	—	—	—
CEA	—	—	+	+	NT	— and +*	— and +*
p53	—	—	—	—	NT	— and +*	— and +*
PCNA	—	—	+	+	—	+	+
Ki-67	—	—	+	+	—	—	—
Blood group A	—	—	+	+	+	—	—
Blood group B	—	—	—	+	+	—	—
Blood group H	—	—	+	+	—	—	—
Apo-A	—	—	+	—	+	—	—
β Catenin	—	—	—	—	—	—	—
Biotin	±	±	±	—	±	NT	NT

*3 cases are positive.

A, pulmonary blastoma; Apo, apolipoprotein; B, endometrioid carcinoma; C, papillary carcinoma of the lung; CEA, carcinoembryonic antigen; D, papillary carcinoma of the thyroid; E, well differentiated fetal adenocarcinoma of the lung; EMA, epithelial membrane antigen; GFAP, glial fibrillary acidic protein; NSE γ , human neurone specific enolase γ ; NT, not tested; PCNA, proliferative cell nuclear antigen; SCC, squamous cell carcinoma; —, no cells react with antibody, ±, a few cells are positive for antibody; +, most cells are positive for antibody; (+), a few cancer cells are positive.

according to the method of Paracios and colleagues⁸ and Kajino *et al.*¹⁶ PCR products of 200 bp at exon 3 where the mutations were reported^{5, 8, 16} were analysed using the LI-COR DNA sequencer, Model 4200 (LI-COR Inc, Lincoln, Nebraska, USA).

RESULTS

Morphological findings

The morules consisted of uniform spindle shaped or round cells with faint eosinophilic cytoplasm and small round nuclei. They filled the lumen of the glandular structures of the carcinomas, and seemed to be formed from the glandular epithelium. Occasionally, they were formed directly from stromal tissue, and had pushed up into the epithelial carcinoma cells (figs 1A, 2A) or intermingled with them. There were no nuclear mitoses. Morules in the pulmonary blastoma showed some optically clear nuclei (fig 1B, C), although the shapes of the cells and nuclei were similar to those in the ECs. The two PBs consisted of EC-like components with morules and exhibited primitive mesenchymal proliferation.

Although we examined three cases each of nephroblastoma, neuroblastoma, medulloblastoma, and ameloblastoma, and one case each of mixed germ cell tumour of the testis and ovarian yolk sac tumour, no morules were found.

In three cases of WDFA of the lung cellular nodules closely resembling morules were found (fig 1D). However, the nuclei were more atypical and slightly larger than those of the morules seen in PB. Small numbers of optically clear nuclei were found in these cellular nodules. They filled the lumen of the glandular structure of the carcinoma and were formed from glandular cancer cells. We refer to them below as morule-like features.

Many morule-like features were found in papillary carcinoma of the thyroid (fig 1E). The tumour cells in the three cases of papillary carcinoma of the lung showed a glandular proliferation with a papillary pattern along with fibrovascular cores, and the tumours were diagnosed as

typical papillary adenocarcinomas of the lung. Fetal adenocarcinoma and endometrioid carcinoma patterns were not demonstrated. There were considerable numbers of morule-like nodules, which were rounded, and solid epithelial cell collections consisting of squamoid spindle shaped cells with faint eosinophilic cytoplasm and small round nuclei (fig 1F). However, neither nuclear mitotic figures nor optically clear nuclei were demonstrated.

The morule cells did not react with antibodies to epithelial cell markers (figs 1A, 2A). The epithelial cancer cells, which were stained clearly with antibodies to epithelial cell antigens, were pushed up by the growth of morules into the glandular lumens and were found on the margin of the morules (figs 1A, 2A). Cancer cells immunopositive for the epithelial antigens, carcinoembryonic antigen, and other tumour markers were occasionally intermingled with morules, and were found in them under certain cutting conditions. Although the morules in PB did not react with antibodies for epithelial markers or surfactant (apolipoprotein 1), they were positive for chromogranin, synaptophysin, enkephalin, and somatostatin (fig 2B). However, NSE γ was not positive in morules in this PB case. Furthermore, carcinoembryonic antigen, p53, proliferative cell markers such as proliferative cell nuclear antigen and c-kit were not positive. β Catenin staining was not seen in the nuclei, although a few cancer cells were positive at the cell membrane. In contrast, the morules in endometrioid carcinoma were positive for NSE γ (fig 2C), although neither neuropeptides nor oncofetal antigens were demonstrated.

In contrast, epithelial cell markers, cytokeratins, involucrin, and epithelial membrane antigen were positive in the morule-like features of three cases of WDFA (fig 1D), in three cases of papillary carcinoma of the lung (fig 2D), and in one case of thyroid carcinoma, in addition to the control squamous cell carcinoma and squamous cell carcinoma components of the adenosquamous carcinoma of the lung. In the morule-like features of thyroid carcinoma, proliferative cell antigens and oncofetal antigens such as blood group B

Figure 2 (A) Pulmonary blastoma. Immunohistochemical demonstration of cytokeratin AE1/AE3. Cancer cells (arrowheads) are strongly positive for AE1/AE3, but morules (asterisks) are negative. The cancer cells (arrowheads) are pushed up by the morule into the glandular lumen and seen on the margin of the morule. Original magnification, $\times 150$. (B) Pulmonary blastoma. Immunostaining for synaptophysin. Staining for synaptophysin (arrowheads) is strong in the morule. Original magnification, $\times 150$. (C) Uterine endometrioid carcinoma. Immunostaining for neurone specific enolase γ (NSE γ). NSE γ (arrowheads) staining is strong in the morules. Original magnification, $\times 150$. (D) Papillary carcinoma of the lung. Immunohistochemical demonstration of epithelial membrane antigen (EMA). Papillary carcinoma cells and a morule-like feature (asterisk) are immunopositive for EMA. Original magnification, $\times 150$.

Figure 3 (A) Sequence analysis of the β catenin gene in a pulmonary blastoma case. The mutation at 315 (G→T) is indicated by the arrow and is associated with an amino acid change (Gly34Val). (B) Sequence analysis of the β catenin gene in a well differentiated fetal adenocarcinoma case. A mutation at 312 (C→G) is indicated by the arrow, and is associated with an amino acid change (Ser33Cys). (C) Sequence analysis of the β catenin gene. The non-mutated wild-type sequence was found in endometrioid carcinoma.

(data not shown) were strongly positive. Furthermore, in WDFAs of the lung, morule-like features were not positive for endocrine peptides.

HPV DNA was not found in either the morules or the morule-like features.

Sequence analysis of β catenin gene

A heterozygous substitution mutation of the β catenin gene was demonstrated at the glycogen synthase kinase 3 phosphorylation site in the morules of one case each of PB (Glu34Val) and EC (Ser33Cys) (fig 3A), and in two cases of W DFA of the lung (Ser33Cys) (fig 3B). The mutation was not seen in the other ECs and W DFA of the lung (fig 3C). The β catenin gene was not analysed in the thyroid carcinomas and one case of PB because DNA could not be obtained.

DISCUSSION

An endocrine cell derivation of morules in PB and adenocarcinoma of the fetal lung has been reported previously, where morules are composed of argyrophilic cells and neurosecretory peptides, such as somatostatin and enkephalin positive cells.¹⁻⁵ The morules in the EC-like component in our PB cases also showed similar neuroendocrine differentiation, with optically clear nuclei. The optically clear nuclei in morule cells were found to be rich in biotin, as reported previously.^{4,5} Moreover, morules were distinct from pulmonary neuroendocrine cell hyperplasia, which was found at the bronchiolar wall, and often replaced the bronchiolar epithelium. PB is a very rare tumour, and shows a unique combination of a primitive glandular epithelium resembling EC with morules and primitive mesenchymal proliferation.

Morules are occasionally seen in uterine EC. They have been considered to be immature squamous cell nodules. We have recently reported that these morules are completely different from squamous differentiation tissue, and that they do not react with epithelial cell markers, but are positive for NSE γ .¹⁴

In contrast, morule-like features are seen in association with papillary carcinoma of the lung and thyroid in rare instances, and with WDFAs. Morule-like features are distinguishable both histologically and immunohistochemically from morules in PB of the lung and ECs. Although β catenin gene mutation has been reported in morules,^{5,7} this was not found in our cases with morule-like features. Miyake *et al* also reported the absence of this mutation in thyroid carcinomas.¹⁷ β Catenin gene mutation was seen in one case each of EC and PB, and in two WDFAs of the lung. In previous reports,^{5,14} and in our present study, both wild-type and mutated alleles were noted. There was no clear association between β catenin gene mutation and morule formation in EC, but there was an association in PB, as reported by Nakatani *et al* and Sekine *et al*.^{5,7}

To the best of our knowledge, there have been no previous reports of morule-like features as distinct from morules

associated with either papillary carcinoma, W DFA of the lung, or thyroid papillary carcinoma. Fornelli *et al* reported a morule-like feature associated with bronchioloalveolar carcinoma, which showed a strong positive reaction to cytokeratin AE1/AE3, and was negative for chromogranin and synaptophysin.⁹ The peculiar morule-like features were considered to be multiple foci of nodular growth of cancer cells. Care should be taken to distinguish clearly between morule-like features and actual morules.

“There was no clear association between β catenin gene mutation and morule formation in endometrioid carcinoma, but there was an association in pulmonary blastoma”

Morule formation has been reported in W DFA of the lung.¹ However, morules were not found in the three cases reported here. Biotin containing optically clear nuclei were found sporadically in the morule-like features in our carcinoma cases. Such nuclei have been demonstrated previously in endometrial glands during gestation, and in morule-like features in a colonic tubular adenoma,¹¹ and were thought to be related to sex hormone functions¹⁸ and hyperglyconeogenesis.¹⁹

Although it is well known^{1,3,4,16} that morules are found in PB and uterine EC, and occasionally in benign endometrial lesions, such as chronic endometritis and hyperplasia, the reason for their formation is unclear. Based on our study, it is possible that morules associated with EC that express NSE γ ¹⁴, and in a few cases acetylcholine esterase, are formed in response to the contraction of the uterus as pressure receptor-like cell clusters. However, the morules found in PBs were immunopositive for neuropeptides. Their function is probably different from those in the

Take home messages

- Morules in pulmonary blastoma and uterine endometrioid carcinoma were non-epithelial cell clusters showing neuronal differentiation
- There were two different types of morule: endometrioid-type morules, which expressed neurone specific enolase γ , and blastoma-type morules, which expressed neuropeptides
- Morule-like features, which closely resemble morules histopathologically, were seen in well differentiated fetal adenocarcinoma of the lung and other carcinomas, and were found to be epithelial nodules
- Although the number of cases was small, the presence of morules showed no clear prognostic correlations

endometrium. It might be useful to classify morules into two groups: neuroendocrine type (blastoma type), which express neuropeptides, and neuroectodermal type (endometrioid type), which express NSE γ and/or acetylcholine esterase.

However, there were only two pulmonary blastomas, and the biological characteristics and functions of the morules are still obscure. In the lung tumours analysed here, the morules were negative for NSE, but Mardini *et al* reported that their samples were positive for NSE and showed carcinoid-like features.²

Furthermore, the squamoid nodules previously reported¹⁹⁻²¹ in pancreatoblastoma might be classified as morules, and they were immunonegative for epithelial cell markers.

In addition, there was no clear association between HPV infection and morules or morule-like features, although HPV had been postulated to be a possible cause of squamous differentiation.¹⁴ In conclusion, the morules were non-epithelial cell clusters showing neuronal differentiation. In contrast, similar morule-like features were indeed epithelial nodules. Adenocarcinomas with morules were different from those with squamous differentiation foci, and there seemed to be no clear prognostic correlation with the presence of morules in carcinomas, although the number of cases is too small to be given statistical weight.

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