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**Case Report**

# Spontaneous Adenosquamous Carcinoma with Rapid Growth and EMT-like Changes in the Mammary Gland of a Young Adult Female BALB/c Mouse

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**Abstract:** This study histopathologically and immunohistochemically investigated a spontaneously occurring single mass subcutaneously located in the left lower abdomen of a female BALB/cAJcl−*nu*/+ mouse at 10 weeks of age. The mass was about 20 × 15 × 10 mm in size after formalin fixation; nevertheless, it was not detected by clinical observations at 9 weeks of age. H&E staining revealed the tumor origin was epithelial and probably arose from the mammary gland, and the tumor cells demonstrated a squamous, acinar or polyhedral/basal pattern. A cell kinetics analysis revealed that many of the tumor cells of the squamous, acinar or polyhedral/basal component were positive for PCNA and cyclin D1, although there were a few of TUNEL-positive tumor cells in all of the components. An epithelial/mesenchymal analysis demonstrated that most of the tumor cells of the squamous and acinar components contained keratin and E-cadherin; however, most of the tumor cells of the polyhedral/basal component were less or very weakly positive for these markers. The tumor cells of the squamous component were negative for vimentin and SMA; however, many of the tumor cells of the polyhedral/basal component exhibited vimentin. In addition, expression of SMA was confirmed in some tumor cells of the acinar and basal components. Based on the microscopic and immunohistochemical characterizations, the tumor was diagnosed to be adenosquamous carcinoma that originated from the mammary gland with rapid growth, and the tumor cells demonstrated epithelial-mesenchymal transition-like changes. (DOI: 10.1293/tox.25.265; J Toxicol Pathol 2012; 25: 265–271)

**Key words:** adenosquamous carcinoma, rapid growth, mouse, immunohistochemistry

A spontaneous female mammary neoplasm in mice of CH3 and DBA/Z strains that carry the mouse mammary tumor virus is common, however, that of BALB/c, CD-1 and B6C3F1 strains mainly used in toxicity or carcinogenicity study, is not major finding even in aged animals[1–5]. In the case of female CD-1 mice, the earliest identified lethal malignant mammary tumor has been reported at from 41−45 weeks in carcinogenicity studies[6], and so far as we know, there are no reports of spontaneous mammary tumor in young adult mice of any strains with detailed analyses using cell kinetics markers as well as epithelial/mesenchymal markers.

We found a spontaneously occurring subcutaneous mass with rapid growth located in the lower abdomen of a female BALB/c mouse at 10 weeks of age and histopathologically and immunohistochemically investigated the characteristics of this tumor.

The animal was a female BALB/cAJcl−*nu*/+ mouse obtained from CLEA Japan, Inc. (Tokyo, Japan) at 5 weeks of age and was placed in an environment/infection monitoring group (no treatments were planned). The mouse was kept in group of three mice in a flat-bottom plastic cage with paper bedding (about 415 $cm^2$ with a height of 13 cm) and housed in a barrier-sustained room maintained at a temperature of 19–25°C with a relative humidity of 30–70%, a 12 hour light-dark cycle and free access to a commercial diet (FR−2, Funabashi Farm Co., Ltd., Chiba, Japan) and tap water. The mouse was checked twice daily by cage-side observation and underwent individual detailed clinical examination and body weight measurement weekly. The detailed clinical examination at 10 weeks of age revealed a subcutaneous mass in the left lower abdomen (in contact with the vulva) of the animal with a skin ulcer, and the mouse was subsequently housed individually. No abnormalities were detected in any

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**Table 1.** Primary Antibodies and Key Reagents Used for Immunohistochemical Procedures

| Primary antibody | Antigen retrieval | Protein block and visualization |
|---|---|---|
| *Cell kinetics markers* | | |
| Monoclonal mouse anti-PCNA<br>Clone PC10, code N1529, Dako Denmark A/S, Glostrup, Denmark | Microwave at 500W (20 min); in Target Retrieval Solution, pH 9, code S2367, Dako Denmark A/S | Histfine® MOUSESTAIN Kit, code 414322, Nichirei Corporation, Tokyo, Japan |
| Monoclonal mouse anti-cyclin D1<br>Clone SP4, code 413521, Nichirei Corp., Tokyo, Japan | Microwave at 500W (20 min); in Target Retrieval Solution, pH 9, code S2367, Dako Denmark A/S | Histfine® MOUSESTAIN Kit, code 414322, Nichirei Corporation |
| *Epithelial/ mesenchymal markers* | | |
| Monoclonal mouse anti-keratin/cytokeratin<br>Clone AE1 and AE3, code 412811, Nichirei Corp. | Proteinase K (15 min), code S3020, Dako Denmark A/S | Histfine® MOUSESTAIN Kit, code 414322, Nichirei Corporation |
| Monoclonal mouse anti-E-cadherin<br>Clone NCH−38, code N1620, Dako Denmark A/S | Microwave at 500W (20 min); in Target Retrieval Solution, pH 9, code S2367, Dako Denmark A/S | Histfine® MOUSESTAIN Kit, code 414322, Nichirei Corporation |
| Monoclonal rabbit anti-vimentin<br>Clone EPR3776, code ab92547, Abcam plc, Cambridge, UK | Microwave at 500W (20 min); in Target Retrieval Solution, pH 9, code S2367, Dako Denmark A/S | Protein Block, code X0909, Dako Denmark A/S<br>EnVision® +System−HRP Labelled Polymer Anti-Rabbit; code K4003, Dako Denmark A/S |
| Monoclonal mouse anti-SMA<br>Clone ASM−1, code 61001, PROGEN Biotechnik GmbH, Heidelberg, Germany | Microwave at 500W (20 min); in Target Retrieval Solution, pH 9, code S2367, Dako Denmark A/S | Histfine® MOUSESTAIN Kit, code 414322, Nichirei Corporation |
| *Negative control antibodies* | | |
| N-universal negative control mouse<br>Code N1698, Dako Denmark A/S | Microwave at 500W (20 min); in Target Retrieval Solution, pH 9, code S2367, Dako Denmark A/S | Histfine® MOUSESTAIN Kit, code 414322, Nichirei Corporation |
| N-universal negative control rabbit<br>Code N1699, Dako Denmark A/S | Microwave at 500W (20 min); in Target Retrieval Solution, pH 9, code S2367, Dako Denmark A/S | Protein Block, code X0909, Dako Denmark A/S<br>EnVision® +System−HRP Labelled Polymer Anti-Rabbit; code K4003, Dako Denmark A/S |

of the previous examinations. The mouse was euthanized by exsanguination under deep inhalational anesthesia using isoflurane (Mylan Inc., Canonsburg, PA, USA) three days after the detailed clinical observation. All experiments were conducted in accordance with the Standards for Proper Conduct of Animal Experiments, Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan).

An autopsy revealed a single mass (about 20 × 15 × 10 mm in size after formalin fixation) subcutaneously located in the left abdomen. The left clitoral gland was disappeared and replaced by the tumor mass. No visible masses/nodules or apparent abnormalities were observed in the other organs/tissues. The mass was removed, fixed in phosphate-buffered 10% neutral formalin, embedded in paraffin and sectioned (5 μm thick). One of the sections was stained with hematoxylin and eosin (H&E).

In addition, the other sections were stained for cell kinetics markers [immunohistochemistry for proliferating cell nuclear antigen (PCNA) and cyclin D1 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)] and epithelial/mesenchymal markers [immunohistochemistry for keratin, E-cadherin, vimentin and smooth muscle actin (SMA)], respectively. The primary antibodies and key reagents used for immunohistochemistry are listed in Table 1. An ApopTag® peroxidase in situ apoptosis detection kit was used for the TUNEL method (code: S7100, Millipore Corporation, Billerica, MA, USA). The sections were developed with a diaminobenzidine hydrogen peroxidase substrate and counterstained with hematoxylin.

All sections were examined under a light microscope.

The mass was slightly encapsulated and showed a multilobular pattern divided by thin connective tissue. A necrotic area was recognized in the center of the mass. The tumor cells demonstrated a squamous, acinar or polyhedral/basal pattern, and a linkage structure connecting to all 3 types of tumor cells was confirmed (Fig. 1A). Spindle or spindloid type tumor cells were not detected. The squamous phenotype tumor cells were considered to be well differentiated and formed cavities of different sizes with some mitotic figures (Figs. 1B and 1E), and they were dominant in the deeper areas around the necrosis (Fig. 1A). The acinar phenotype tumor cells were considered to be moderately to well differentiated and formed a glandular-like structure (Figs. 1C and 1F). The polyhedral/basal phenotype tumor cells that resembled the basal keratinocyte were considered to be poorly differentiated and formed nests of solid growth (Figs. 1D and 1G). Both the acinar and polyhedral/basal phenotype tumor cells had large round to oval nuclei with frequent mitotic figures and scant eosinophilic cytoplasm. They were dominant in the superficial area and invaded the adjacent/surrounding subcutaneous tissue and skin (Fig. 1A). Based on these microscopic features of the H&E stain section, the

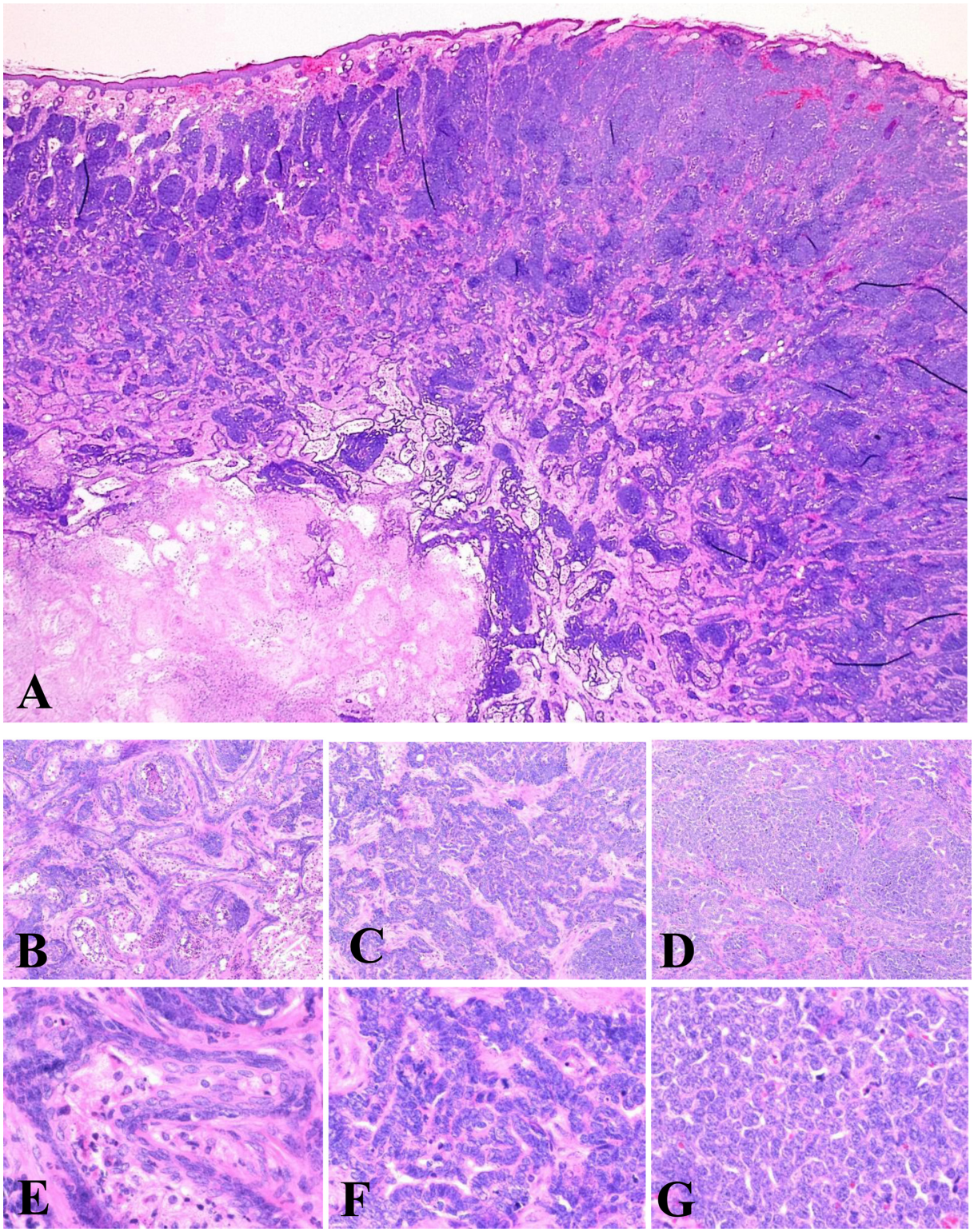


**Fig. 1.** Histopathological appearance of the tumor (A). The mass had a necrotic area in the center, and the vital tumor cells demonstrated squamous (B and E), acinar (C and F) and polyhedral/basal (D and G) patterns. Cells that exhibited the acinar and polyhedral/basal patterns were dominant in the superficial area of the mass and invaded the adjacent/surrounding subcutaneous tissue. H&E staining. Original magnification: ×1.4 (A), ×10 (B, C and D) and ×40 (E, F and G).

mass was suggested a single malignant tumor of epithelial origin. Although the left clitoral gland was not found macroscopically, the mass was suggested to be arose from the mammary gland because no evidence of differentiation of the tumor cells to a sebaceous-like or hair follicle-like structure was observed. Therefore, the mass was diagnosed to be

**Table 2.** Summary of Immunohistochemistry and TUNEL Method Results

| Method | Type of the tumor cell | | |
|---|---|---|---|
| | Squamous | Acinar | Polyhedral/Basal |
| PCNA IHC | 2+ | 3+ | 3+ |
| Cyclin D1 IHC | 3+ | 3+ | 3+ |
| TUNEL | 1+ | 1+ | 1+ |
| Keratin/cytokeratin IHC | 4+ | 4+ | − |
| E-cadherin IHC | 4+ | 4+ | 2+ |
| Vimentin IHC | − | − | 3+ |
| SMA IHC | − | 2+ | 2+ |

IHC = immunohistochemistry. −, negative / no apparent positive cells; 1+, a few positive cells; 2+, some positive cells; 3+, many positive cells; and 4+, most cells positive.

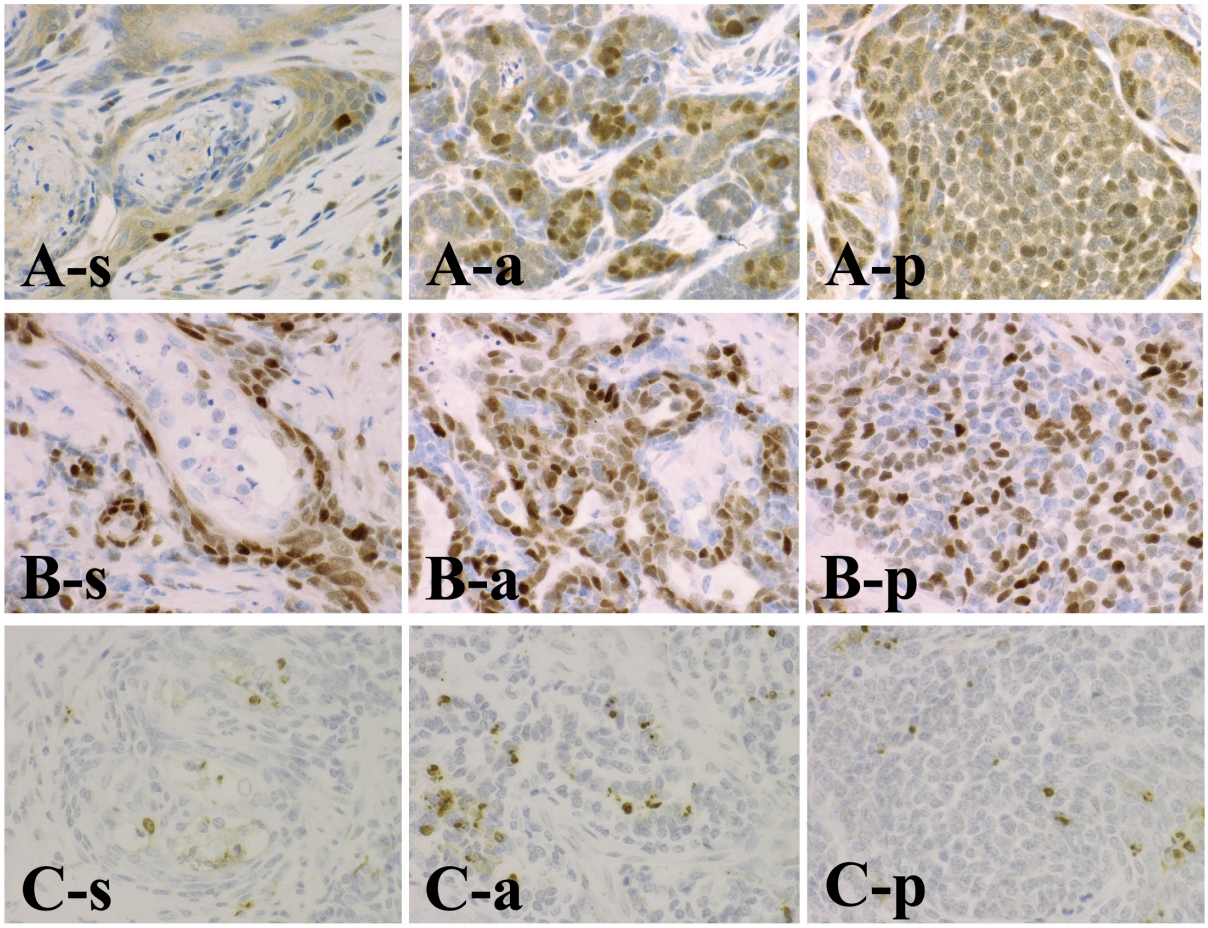


**Fig. 2.** Immunohistochemistry for PCNA (A) and cyclin D1 (B) and the TUNEL method (C). Nuclei of many tumor cells of the each component demonstrated a specific positive reaction for PCNA and cyclin D1, although there were a small number of TUNEL-positive tumor cells in all components. Original magnification: ×40. s: squamous component. a: acinar component. p: polyhedral/basal component.

adenosquamous carcinoma (adenoacanthoma) of the mammary gland.

The results of cell kinetics and epithelial/mesenchymal analyses are summarized in Table 2. Many of the tumor cells in the squamous, acinar or polyhedral/basal component were positive for cell proliferative markers (PCNA and cyclin D1; Figs. 2A and 2B). There were a few apoptotic tumor cells (TUNEL-positive tumor cells) in all of the components (Fig. 2C). Most of the tumor cells in the squamous and acinar components exhibited keratin and E-cadherin, while most tumor cells of the polyhedral/basal component showed no or very weak staining for these markers (Figs. 3A and 3B). The cells in the squamous component were negative for vimentin and SMA; however, many tumor cells in the polyhedral/basal component, as well as mesenchymal cells in the interstitium, exhibited vimentin (Fig. 3C). In ad-

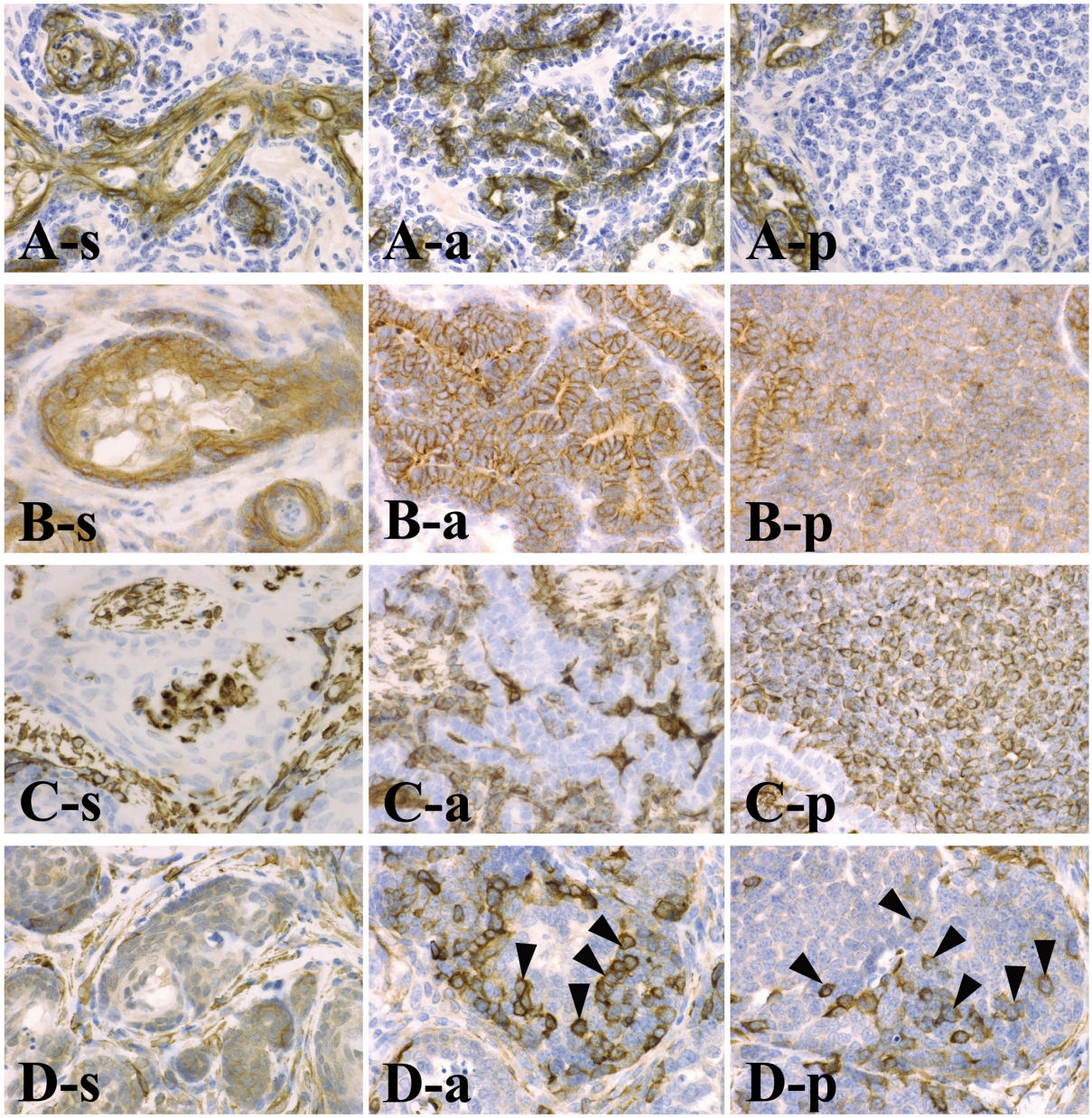


**Fig. 3.** Immunohistochemistry for keratin (A), E-cadherin (B), vimentin (C) and SMA (D). The cytoplasm or cell membrane of most tumor cells of the squamous and acinar components demonstrated a specific positive reaction for keratin/cytokeratin (A-s and A-a) and E-cadherin (B-s and B-a). However, there was little or very weak staining in most of the tumor cells in the basal component (A-p and B-p). The cytoplasm of mesenchymal cells in the interstitium was positive for vimentin and SMA. However, the tumor cells in the squamous component were negative (C-s and D-s), although the cytoplasm of many tumor cells in the basal component showed a specific positive reaction for vimentin (C-p), and some tumor cells (arrow heads) in the acinar and polyhedral/basal components showed a specific positive reaction for SMA (D-a and D-p). Original magnification: ×40.

dition, there was expression of SMA in some tumor cells located in the central region of tumor cell lobules/nests of the acinar and polyhedral/basal components (Fig. 3D, arrow heads). There were also some positive cells for SMA in the peripheral regions of the lobules/nests; however, these cells could not be discriminated from the myoepithelial cells adjacent to the lobules/nests (Fig. 3D). The sections treated with control primary antibodies showed no specific positive reactions.

There was prominent cellular proliferation, characterized by the PCNA- and cyclin D1-positive tumor cells, especially in the acinar and polyhedral/basal components and a small number of apoptotic tumor cells. These were considered to have lead to very rapid growth of the tumor after only about 1 week. Overexpression of cyclin D1 has been observed in various human malignant tumors[7–12] and in murine chemical carcinogenesis[13–17] and is thought to be as a factor associated with malignant transformation. Therefore,

the overexpression of cyclin D1 in this spontaneous tumor was also thought to be associated with malignant progression and invasion of the tumor cells.

The tumor cells exhibited 3 cellular phenotypes, squamous (well-differentiated cells), acinar (well- or moderately differentiated cells) and polyhedral/basal (poorly differentiated cells) patterns. The keratin and E-cadherin expressions both in the squamous and acinar phenotype tumor cells strongly supported that the origin of this tumor would be the epithelium, such as from acinar or ductal cells but not myoepithelial cells. Vimentin expression was confirmed in most tumor cells with the polyhedral/basal phenotype, and SMA was observed in some acinar and polyhedral/basal phenotype tumor cells. Kobayashi *et al.* reported a case of basal cell carcinoma of the submandibular gland in a female Wistar rat at 10 weeks of age with many PCNA-positive tumor cells but no expression of vimentin and SMA[18]. However, Nishikawa *et al.* reported a case of poorly differentiated salivary gland carcinoma in a male SD rat at 10 weeks of age with frequent mitotic figures and positive staining for both keratin and vimentin but not for SMA[19]. Sarrió *et al.* reported that occurrence of epithelial-mesenchymal transition (EMT)-like changes in human breast cancers. The transition was associated with upregulation of mesenchymal markers (such as vimentin and SMA) together with downregulation of epithelial markers (such as cytokeratin and E-cadherin) in cells with a basal-like phenotype, and this expression was thought to contribute the aggressiveness and metastatic spread of the these cancers[20]. The downregulation of epithelial markers and upregulation of mesenchymal markers have been recognized to be essential biomarkers of EMT, which plays a sinister role in tumor progression[21, 22]. Particularly, the repression of E-cadherin is noted to be an important marker for the loss of the epithelial phenotype[21]. The tumor cells in the current tumor, especially those with the polyhedral/basal phenotype, resemble cells undergoing EMT, and this could play a key role in the malignant progression and spread of this tumor. Regarding the mouse mammary tumorigenesis *in vivo*, it has been reported that the expression of mesenchymal characteristics in tumor cells is detected in the spindle− or spindloid−phenotype, but not in the acinar− or basal−phenotype[23–25]. Therefore, the presented case may be the first report to describe a case in which EMT-like changes were detected in non−spindle/spindloid phenotype tumor cells.

In conclusion, the tumor was diagnosed to be adenosquamous carcinoma of the mammary gland with rapid growth, and the tumor cells were thought to demonstrate EMT−like changes.

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