Tumor budding of cervical squamous cell carcinoma: Epithelial–mesenchymal transition-like cancer stem cells?

Shaoqiu Zheng [Corresp., 1] , **Jing Luo** [2] , **Shoucheng Xie** [1] , **Shanming Lu** [1] , **Qinghua Liu** [1] , **Huanqin Xiao** [1] , **Wenjuan Luo** [1] , **Yanfang Huang** [1] , **Kun Liu** [1]

[1] Department of Pathology, Meizhou People's Hospital, Meizhou, Guangdong, China

[2] Department of Pelvic Radiotherapy, Meizhou People's Hospital, Meizhou, Guangdong, China

Corresponding Author: Shaoqiu Zheng
Email address: zhengshaoqiu@mzrmyy.com

Recent evidence indicates that cancer stem cells (CSCs) are the origin of cancers. Scientists have identified CSCs in various tumors and have suggested the existence of a variety of states of CSCs. The existence of epithelial–mesenchymal transition (EMT)-like CSCs has been confirmed *in vitro*, but they have not been identified *in vivo*. Tumor budding was defined as single cell or clusters of ≤ 5 cells at the invasive front of cancers. Such tumor budding is hypothesized to be closely related to EMT and linked to CSCs, especially to those migrating at the invasive front. Therefore, tumor budding has been proposed to represent EMT-like stem cells. However, this hypothesis has not yet been proven. Thus, we studied the expression of EMT markers, certain CSC markers of tumor budding, and the tumor center of cervical squamous cell carcinoma (CxSCC). We performed tissue chip analyses of 95 primary CxSCCs from patients. Expression of EMT and CSC markers (E-cadherin, β-catenin, vimentin, Ki67, CD44, SOX2 , and ALDH1A1) in a set of tumor samples on tissue chips (87 cases of tumor budding/the main tumor body) were evaluated by immunohistochemistry. We found that the cell-membranous expression of β-catenin was stronger in the main tumor body than in tumor buds. Compared with the main tumor body, tumor buds had reduced proliferative activity as measured by Ki67. Moreover, vimentin expression was high and E-cadherin expression was low in tumor buds. Expression of EMT-related markers suggested that tumor buds were correlated with EMT.

We noted that CxSCC tumor buds had a CD44$^{\text{negative/low}}$/SOX2$^{\text{high}}$/ALDH1A1$^{\text{high}}$ staining pattern, indicating that tumor buds of CxSCC present CSC-like immunophenotypic features. Taken together, our data indicate that tumor buds in CxSCC may represent EMT-like CSCs *in vivo.*

1   **Tumor budding of cervical squamous cell carcinoma: Epithelial–mesenchymal transition-**

2   **like cancer stem cells?**

4   Shaoqiu Zheng[1], Jing Luo[2], Shoucheng Xie[1], Shanming Lu[1], Qinghua Liu[1], Huanqin Xiao[1],

5   Wenjuan Luo[1], Yanfang Huang[1], Kun Liu[1]

7   [1]Department of Pathology, Meizhou People's Hospital, Meizhou, Guangdong, China

8   [2]Department of Pelvic Radiotherapy, Meizhou People's Hospital, Meizhou, Guangdong, China

10   **Correspondence to:**

11   Dr. Shaoqiu Zheng

12   Department of Pathology, Meizhou People's Hospital, No. 63 Huangtang Road, Meizhou,

13   Guangdong 514031, China

14   E-mail: zhengshaoqiu@mzrmyy.com

29 **Abstract**

30 **Background.** Recent evidence indicates that cancer stem cells (CSCs) are the origin of cancers.

31 Scientists have identified CSCs in various tumors and have suggested the existence of a variety

32 of states of CSCs. The existence of epithelial–mesenchymal transition (EMT)-like CSCs has

33 been confirmed *in vitro*, but they have not been identified *in vivo*. Tumor budding has been

34 defined as single cell or clusters of ≤5 cells at the invasive front of cancers. Such tumor budding

35 is hypothesized to be closely related to EMT and linked to CSCs, especially to those migrating at

36 the invasive front. Therefore, tumor budding has been proposed to represent EMT-like stem cells.

37 However, this hypothesis has not yet been evaluated. We aimed to examine the expression of

38 EMT markers, certain CSC markers of tumor budding, and the tumor center of cervical

39 squamous cell carcinoma (CxSCC). **Methodology.** We performed tissue chip analyses of 95

40 primary CxSCCs from patients. Expression of EMT and CSC markers (E-cadherin, β-catenin,

41 vimentin, Ki67, CD44, SOX2, and ALDH1A1) in a set of tumor samples on tissue chips (87

42 cases of tumor budding/the main tumor body) was evaluated using immunohistochemistry.

43 **Results.** We found that the cell-membranous expression of β-catenin was stronger in the main

44 tumor body than in tumor buds. Compared with the main tumor body, tumor buds had reduced

45 proliferative activity as measured using Ki67. Moreover, vimentin expression was high and E-

46 cadherin expression was low in tumor buds. Expression of EMT-related markers suggested that

47 tumor buds were correlated with EMT. **Conclusion.** CxSCC tumor buds had a

48 CD44$^{negative/low}$/SOX2^{high}/ALDH1A1^{high} staining pattern, indicating that tumor buds of CxSCC

49 present CSC-like immunophenotypic features. Taken together, our data indicate that tumor buds

50 in CxSCC may represent EMT-like CSCs *in vivo*.

51

52 **Introduction**

53   Tumor budding is considered to be a single or small cluster of tumor cells (≤5 cells) present

54 at the invasive front of cancers. Imai et al. first described tumor budding in 1960 (*Imai, 1960*),

55 and it is accepted as an independent prognostic factor in many cancers, such as colorectal cancer

56   (CRC) (*Lugli, Karamitopoulou & Zlobec, 2012*), pancreatic ductal adenocarcinoma (PDAC)

57   (*O'Connor et al., 2015*), tongue cancer (*Ebihara et al., 2019*), lung cancer (*Kadota et al., 2015*),

58   and cervical cancer (*Park et al., 2020*). Although there have been many studies on tumor

59   budding, only a few have investigated tumor buds in cervical cancer, while mainly focusing on

60   the relationship between tumor buds and the clinicopathological characteristics of cervical cancer,

61   tumor buds, and cervical cancer patient prognosis (*Huang et al., 2016; Satabongkoch et al., 2017;*

62   *Ferrandina et al., 2017; Jesinghaus et al., 2018; Park et al., 2020; Zare et al., 2020; Cao et al.,*

63   *2020; Chong et al., 2021*).

64   Epithelial-mesenchymal transformation (EMT) is a process in which epithelial cells lose

65   adhesion and acquire mesenchymal cell characteristics as well as the ability to migrate and

66   invade (*Kalluri & Weinberg, 2009*). Tumor budding is deemed to reflect the process of EMT

67   (*Karamitopoulou, 2013*). E-cadherin is expressed in low quantities in tumor buds in various

68   cancer types. Low expression of E-cadherin is a hallmark of EMT; thus, tumor buds have been

69   proposed to be "EMT-like" (*Grigore et al., 2016*).

70   Cancer stem cells (CSC) are considered to be the main culprit of tumor local invasion and

71   metastasis (*Marangon et al., 2019*). Tumor budding is known to be a morphological marker of

72   tumor invasion. Additionally, it has been suggested that tumor buds may have "stem cell"

73   characteristics (*Marangon et al., 2019*). In our study, we found an association between tumor

74   buds and CSCs. For instance, tumor buds in CxSCC expressed biomarkers of CSCs. However,

75   compared to the main tumor, tumor buds demonstrated reduced proliferative activity. This was in

76   contrast to the unlimited proliferative potential of CSCs. We assumed that the tumor buds

77   represent a transient phase in the lifetime of CSCs. In vitro studies have shown that CSCs and

78   EMT-type cells have overlapping phenotypes. Properties of CSCs and EMT-type cells may be

79   linked together through shared molecular features (*Floor et al., 2011*). Additionally, typical

80   EMT-type cells do not proliferate. We hypothesized that tumor buds were CSCs in an EMT state.

81   Nevertheless, the relationships among tumor budding cells, EMT-type cells, and CSCs

82   remain controversial. In this study, we aimed to investigate EMT- and CSC-like molecular

83   characteristics of tumor budding in CxSCC and explore their possible interactions.

84

85   **Materials & Methods**

86   *Patients and tumor tissues*: This study was approved by the Ethics Committee of Meizhou

87   People's Hospital (approval no. 2019-C-51). Written informed consent was obtained from all

88   patients. All procedures were performed according to the World Medical Association's

89   Declaration of Helsinki.

90   Ninety-five patients with CxSCC who underwent radical hysterectomy and pelvic

91   lymphadenectomy from January 2018 to December 2018 at our institution were enrolled. The

92   median patient age was 52 (range, 26–78) years.

93   The staging of all samples was performed according to The International Federation of

94   Gynecology and Obstetrics classification (2009). Of 95 samples, 43 were classified as clinical

95   stage Ib1 and Ib2, and 52 were classified as stages IIa1 and IIa2. Twelve of 95 patients with

96   CxSCC (12.6%) had lymph node metastases, and 45.3% had lymphatic invasion, as shown in

97   Table 1.

98

99   *Immunohistochemical staining of tissue microarray:* Tumor material was paraffin-embedded

100   after fixation in 10% neutral buffered formalin. Representative tumor areas (tumor budding and

101   the main tumor body) were marked on hematoxylin and eosin-stained slides, and 2-mm tissue

102   cores from each tumor were arrayed from the corresponding paraffin blocks into a recipient

103   block using a manual tissue chip instrument. Three to four-micrometer-thick paraffin sections

104   were cut from the array blocks. The first sections were stained with hematoxylin and eosin to

105   confirm validity, and the subsequent sections were used for immunohistochemistry (IHC).

106   The expression of E-cadherin, β-catenin, vimentin, Ki67, CD44, ALDH1A1, and SOX2 was

107   evaluated. Without knowledge of follow-up results, two investigators independently reviewed all

108   IHC slides and recorded immunoreactivity for each lesion using a semi-quantitative scoring

109   system. The final score was a combination of staining intensity and extent. The intensity score

110   was as follows: negative (0); weak (1); moderate (2); and strong (3). The staining extent was

111   defined as: 0, negative; 1, <10%; 2, 10–50%; and 3, >50% positive cells. The total score ranged

112   from 0 to 9. Negative immunoreactivity was defined as a total score of 0. Low immunoreactivity

113   was defined as a total score of 1–4. High immunoreactivity was defined as a total score >4. Ki67

114   expression was evaluated quantitatively, based on the percentage of positively-stained cells: 0–

115   5%, no reaction (–); 6–25%, weak reaction (+); 26–50%, moderate reaction (++); and >50%,

116   intense reaction (+++).

117

118   *Statistical analysis*: After observing the relevance of the staining, all statistical analyses were

119   performed using SPSS statistical software, version 23.0 (IBM SPSS Inc., Armonk, NY, USA).

120   Categorical variables were evaluated using a χ^2 test. *P*-values <0.05 were considered statistically

121   significant.

122

123   **Results**

124   There were no significant differences in patient background between those with tumor

125   budding (n = 87) and those without budding (n = 8) (Table 1).

126   Overall, moderate to strong expression of SOX2 and ALDH1A1 was observed more

127   frequently in tumor buds than in the main tumor body (51.7% vs. 9.2%; 48.3% vs. 20.7%,

128   respectively; both *P* < 0.001). Conversely, in 79 (90.8%) samples, the main tumor body showed

129   negative/low expression of SOX2. Furthermore, 79.3% (69/87) of the main tumor body samples

130   showed negative/low expression of ALDH1A1.

131   The IHC results showed that CD44 expression in the main tumor body was significantly

132   higher than that in the tumor buds (65.5% vs. 16.1%). Moreover, moderate to strong expression

133   of CD44 in the main tumor body and tumor buds occurred in 44.8% and 13.8% of samples,

134   respectively. The difference in CD44 expression between the tumor body and tumor buds was

135   significant ($P < 0.001$).

136   Protein expression of the mesenchymal marker vimentin was increased in the tumor buds,

137   whereas expression of the epithelial marker, E-cadherin, was decreased in the tumor buds when

138   compared with that in the main tumor body (21.8% vs. 3.4%; 23.0% vs. 90.8%, respectively;

139   both $P < 0.05$). The cell-membranous expression of β-catenin was stronger in the main tumor

140   body than in tumor buds (92.0% vs. 33.3%, $P < 0.05$).

141   Seventy-one cases showing tumor budding had no reaction/weak staining for Ki67. In

142   contrast, 94.3% (82/87) of the main tumor body samples showed moderate/intense staining for

143   Ki67. The proliferation index of tumor buds was thus significantly lower than that of the main

144   tumor body.

145   The IHC staining results for the tumor buds and main tumor body are summarized in Table 2

146   and in *Figs. 1–3*.

147

148   **Discussion**

149   EMT-like stem cells have not been identified in tumor tissue to date, but tumor budding has

150   been proposed to represent EMT-like stem cells. In this study, we investigated the relationship

151   between EMT-like stem cells and certain CSC markers in tumor buds and in the tumor body of

152   CxSCC specimens. We found that β-catenin was more strongly expressed in cell membranes of

153   the main tumor body than in those of tumor buds, while Ki67 was less strongly expressed in

154   tumor buds, indicating reduced proliferative activity. Moreover, vimentin expression was high,

155   whereas E-cadherin expression was low in tumor buds. The expression of EMT-related markers

156   indicated a correlation between tumor budding and EMT. CxSCC tumor buds demonstrated a

157   CD44$^{negative/low}$/SOX2^{high}/ALDH1A1^{high} staining pattern, suggesting that CxSCC tumor bud cells

158   have CSC-like phenotypic characteristics. Thus, our data indicated that CxSCC tumor buds may

159   be an *in vivo* representation of EMT-like CSCs.

160   Increasing evidence suggests that CSCs are the origin of cancers and play key roles in cancer

161   recurrence and metastasis. Cervical cancer is a growing global burden for both developing and

162   developed countries. Each year, >500,000 new cases of cervical cancer are reported worldwide,

163   and approximately 250,000 women die from cervical cancer (*Fowler, Maani & Jack, 2021*),

164   making the investigation of cervical CSCs important for global health.

165   CSCs can be identified using CSC-related markers, but these markers are not universal to all

166   tumor types. Furthermore, identification of CSCs based on histological morphology is usually

167   limited in *in vivo* studies.

168   Tumor budding, which has been considered to share similarities with CSCs, is easily

169   recognized in a variety of solid tumors. Both CSCs and tumor buds are present at the tumor's

170   invasive front (*Kodama et al., 2017; Lugli et al., 2017*). High-grade tumor budding is an

171   independent prognostic factor for various solid tumors (*Almangush et al., 2016; Cappellesso et

172   al., 2017; Lawlor et al., 2019; Park et al., 2020; Regmi et al., 2022*) and correlates with lymph

173   node metastasis (*Nakagawa et al., 2013; Takamatsu et al., 2019*) and the depth of tumor invasion

174   (*Yamakawa et al., 2019; Xie et al., 2019*). Nevertheless, we found that the presence or absence of

175   tumor budding did not correlate with any clinicopathological variables (Table 1). Previous

176   studies have shown a similar relationship between CSCs and clinicopathological characteristics

177   (*Lu et al., 2008; Eramo et al., 2008; Chen et al., 2013; Yiming et al., 2015*).

178   Based on the above points, we speculated that the tumor buds might be tumor stem cells. As

179   tumor buds are easy to recognize in tumor tissues and have little morphological variation among

180   differing tumor types, we considered it beneficial to further investigate whether the tumor buds

181   could be shown to be tumor stem cells.

182   Several CSC markers have been evaluated in tumor buds. *Hostettler et al.* (*2010*) found that

183   ABCG5 and EpCAM are expressed in numerous CRC tumor buds. *Masaki et al.* (*2001*) linked

184   elevated levels of tumor budding to membranous CD44 and CD44v6 expression. *Attramadal et

185   al. (2015*) scored the expression of SOX2, CD44, ALDH1, CD24, and OCT3/4 in oral squamous

186   cell carcinomas, and found that CD44 was expressed in tumor buds, whereas SOX2 was

187   expressed at the front of tumor invasion.

188   SOX2 is an important transcription factor for the acquisition and maintenance of stem cell

189   properties and is a well-recognized marker of CSCs including CSCs of cervical squamous cell

190   carcinoma (*Chhabra, 2015*). CD44 and ALDH1A1 are both considered to be typical CSC

191   surface markers that can be used alone or in combination with other markers to isolate or enrich

192   CSCs in a variety of tumor types including cervical cancer (*Liu et al., 2016; Wang et al., 2018;*

193   *Tomita et al., 2018; Tulake et al., 2018*). Therefore, we sought to detect the expression of SOX2,

194   CD44, and ALDH1A1 in CxSCC tumor buds. Consistent with our expectations, we observed

195   high expression of SOX2 and ALDH1A1 in CxSCC tumor buds. This suggested that CxSCC

196   tumor budding had a CSC immunophenotype. Unlike studies undertaken by *Masaki et al.* and

197   *Attramadal et al.,* our study showed that CD44 expression in tumor buds was significantly lower

198   than that in the main tumor body (16.1% vs. 65.5%), which was not consistent with our expected

199   results.

200   Many studies have shown that CD44 is expressed or over-expressed in CSCs (*Leng et al.,*

201   *2018; Rabinovich et al., 2018; Elkashty et al., 2020; Okuyama et al., 2020*). Nevertheless, some

202   studies have indicated heterogeneity in relation to CD44 expression in CSCs (*Vitale et al., 2019*).

203   Chopra et al. (*2019*) observed heterogeneous expression of CD44 in cervical cancer that was rich

204   in CSCs, with both CD44-positive and CD44-negative cells appearing in tumors with high CSCs.

205   Moreover, previous studies have suggested the existence of different CSC states. We

206   hypothesized that heterogeneity in CD44 expression was associated with different CSC

207   phenotypes. The presence of CD44-positive and CD44-negative subpopulations in CSC-

208   expressing cells is not well understood. Fan et al. (*2012*) showed that in CRC, CD44 is lost along

209   the invading margins where tumor buds are located. Additionally, we observed that CxSCC

210   tumor buds had a CD44$^{negative/low}$/SOX2^{high}/ALDH1A1^{high} staining pattern. These findings imply

211   that CSCs and tumor budding are related and suggest that tumor budding could represent a

212   certain stage of CSCs.

213   Furthermore, there is evidence to suggest that tumor budding is associated with EMT-like

214   changes. Tumor budding can be considered to represent EMT *in vivo*. Tumor cells in EMT are in

215   a low or non-proliferative state, whereas tumor buds represent a non-proliferating subpopulation

216   of tumor cells (*Zlobec & Lugli, 2010*). In addition, up-regulation of vimentin expression, down-

217   regulation of E-cadherin expression, and a β-catenin cytoplasmic expression pattern are all

218   considered to be characteristics of EMT (*Cervantes-Arias, Pang & Argyle, 2013; Kaszak et al.,*

219   *2020*). We conducted immunohistochemical analysis to detect the expression of E-cadherin,

220   vimentin, β-catenin, and Ki67, and found that CxSCC tumor buds displayed decreased levels of

221   E-cadherin, Ki67, and membranous β-catenin, and increased levels of vimentin. These findings

222   suggest that CxSCC tumor buds have EMT-like phenotypes.

223   Biddle et al. suggested two distinct phenotypes of CSCs (EMT and non-EMT CSCs) in oral

224   squamous cell carcinoma (*Biddle et al., 2011*). Additionally, in some studies, EMT-like CSCs

225   were found to be present *in vitro* (*Biddle et al., 2011; Liu, Clouthier & Wicha, 2012; Yang et al.,*

226   *2017*). We showed that CxSCC tumor buds expressed CSC markers and presented an EMT-like

227   phenotype.

228   Although our findings suggest that tumor budding involves EMT-like CSCs, our study had

229   some limitations. We investigated the properties of EMT and CSCs of the tumor bud through

230   identifying immunophenotypes, which entailed a protein-level study only, without genomic

231   research and exploration. In follow-up studies, we plan to isolate tumor buds from cervical

232   squamous carcinoma tissue through micro-cutting and identify tumor bud characteristics at the

233   gene level. Furthermore, we plan to isolate CD44$^{negative/low}$/SOX2^{high}/ALDH1A1^{high} cells from

234   various cervical cancer cell lines and compare the EMT and biological functions of this

235   subpopulation with those of other subpopulations within the bulk of the cervical squamous cell

236   carcinoma cells.

237

238   **Conclusions**

239   Our data suggest that CxSCC tumor buds represent EMT-like CSCs *in vivo*, which we

240   consider to be an important observation. Our findings fit well with the "migrating cancer stem

241   cell" concept, which holds that CSCs acquire two phenotypes: one associated with growth and

242   the other associated with migration that is characterized as "transient expression of epithelial to

243   mesenchymal transition-associated genes" (*Biddle et al., 2011*). Moreover, we found further

244   evidence to support the presence of EMT-like CSCs *in vivo*, with direct links to tumor budding.

245   We consider that EMT-like and non-EMT CSCs may become potentially important new targets

246   for therapeutic interventions for cancers.

247

248   **Acknowledgments:**

249   Not applicable.

250

251

252   **Ethics approval and consent to participate**:

253   The present study was approved by the Ethics Committee of Meizhou People's Hospital

254   (approval no.2019-C-51). Written informed consent was obtained from all patients. All

255   procedures were performed according to the World Medical Association's Declaration of

256   Helsinki.

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258   **Patient consent for publication**:

259   Not applicable.

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472   **Figure Legends**

473   *Fig. 1*. **Differences in immunohistochemical (IHC) expression of SOX2, CD44, ALDH1A1,**

474   **vimentin, E-cadherin, β-catenin, and Ki67 between tumor buds and the main tumor body,**

475   **based on chi-square test results.**

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477

478   *Fig. 2*. **Immunohistochemical (IHC) staining results for tumor buds.**

479   Tumor buds that are: (A) strongly positive for SOX2; (B) weakly positive for SOX2; (C) CD44-

480   positive; (D) CD44-negative; (E) ALDH1A1-positive; (F) ALDH1A1-negative; (G) vimentin-

481   positive; (H) vimentin-negative; (I) E-cadherin-positive; (J) E-cadherin-negative; (K) β-catenin

482   cytoplasmic-positive; and (L) β-catenin membrane-positive.

483   IHC staining of tumor buds with Ki67 demonstrates: (M) high proliferation and (N) low

484   proliferation.

485   All images: magnification, 100X

486

487   *Fig. 3*. **Immunohistochemical (IHC) staining results for the main tumor body.**

488   The main tumor body that is: (A) negative for SOX2; (B) positive for SOX2; (C) weakly positive

489   for CD44; (D) strongly positive for CD44; (E) ALDH1A1 negative; (F) ALDH1A1 positive; (G)

490   vimentin negative; (H) vimentin positive; (I) weakly positive for E-cadherin; (J) strongly positive

491   for E-cadherin; (K) positive for cytoplasmic β-catenin; and (L) positive for membrane β-catenin.

492   IHC staining of the main tumor body with Ki67 showing: (M) low proliferation and (N) high

493   proliferation.

494   All images: magnification, 100X

495

Figure 1

Fig. 1. Differences in immunohistochemical (IHC) expression of SOX2, CD44, ALDH1A1, vimentin, E-cadherin, β-catenin, and Ki67 between tumor buds and the main tumor body, based on chi-square test results.
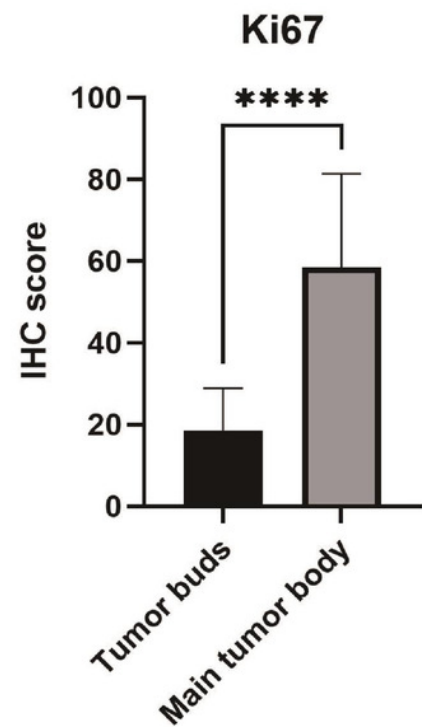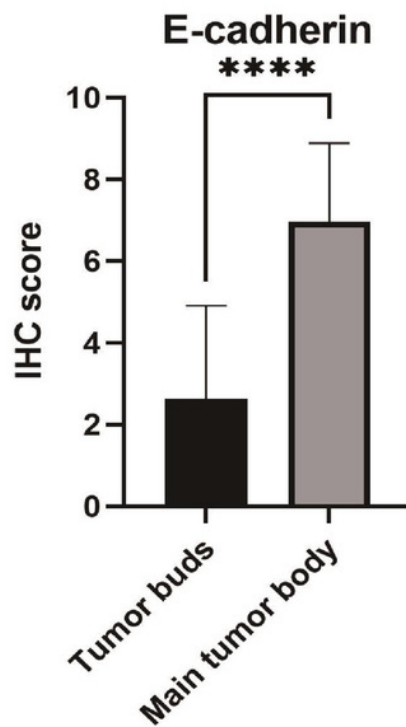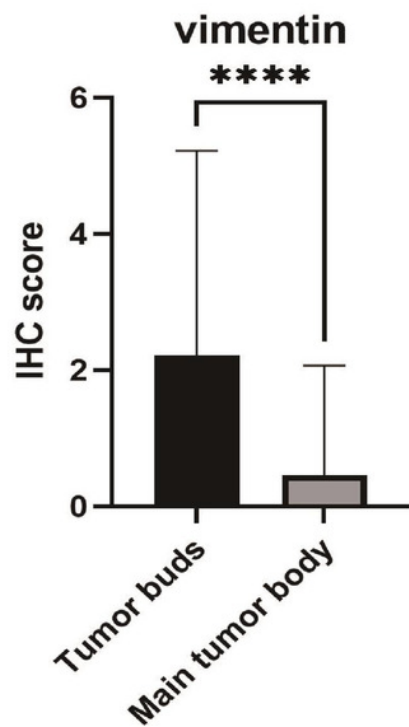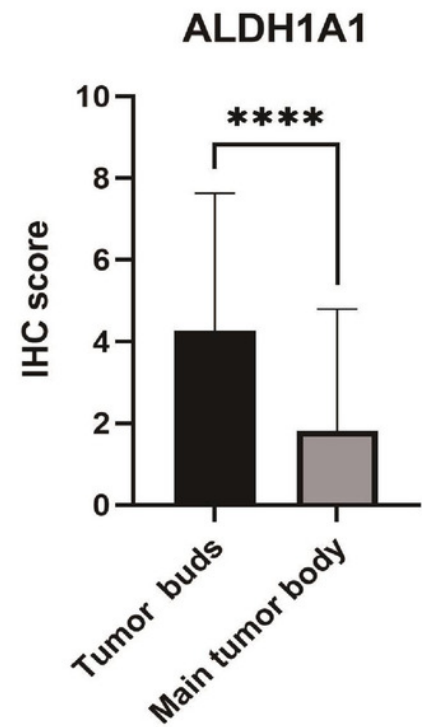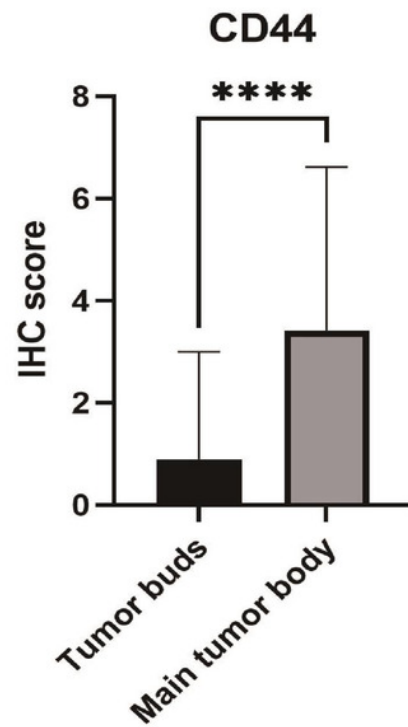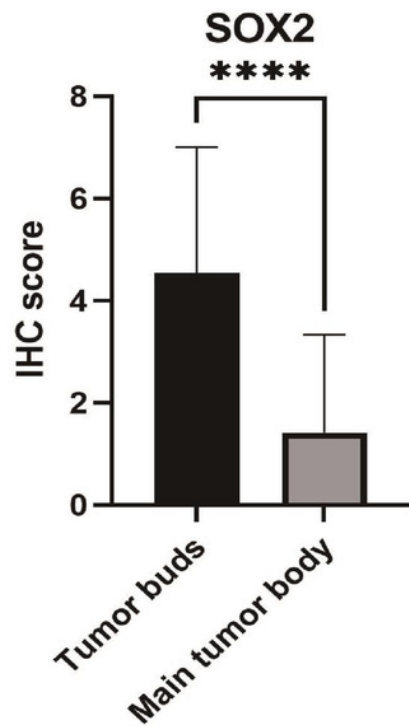
Figure 2

Fig. 2. Immunohistochemical (IHC) staining results for tumor buds.

Tumor buds that are: (A) strongly positive for SOX2; (B) weakly positive for SOX2; (C) CD44-positive; (D) CD44-negative; (E) ALDH1A1-positive; (F) ALDH1A1-negative; (G) vimentin-positive; (H) vimentin-negative; (I) E-cadherin-positive; (J) E-cadherin-negative; (K) β-catenin cytoplasmic-positive; and (L) β-catenin membrane-positive.IHC staining of tumor buds with Ki67 demonstrates: (M) high proliferation and (N) low proliferation.All images: magnification, 100X
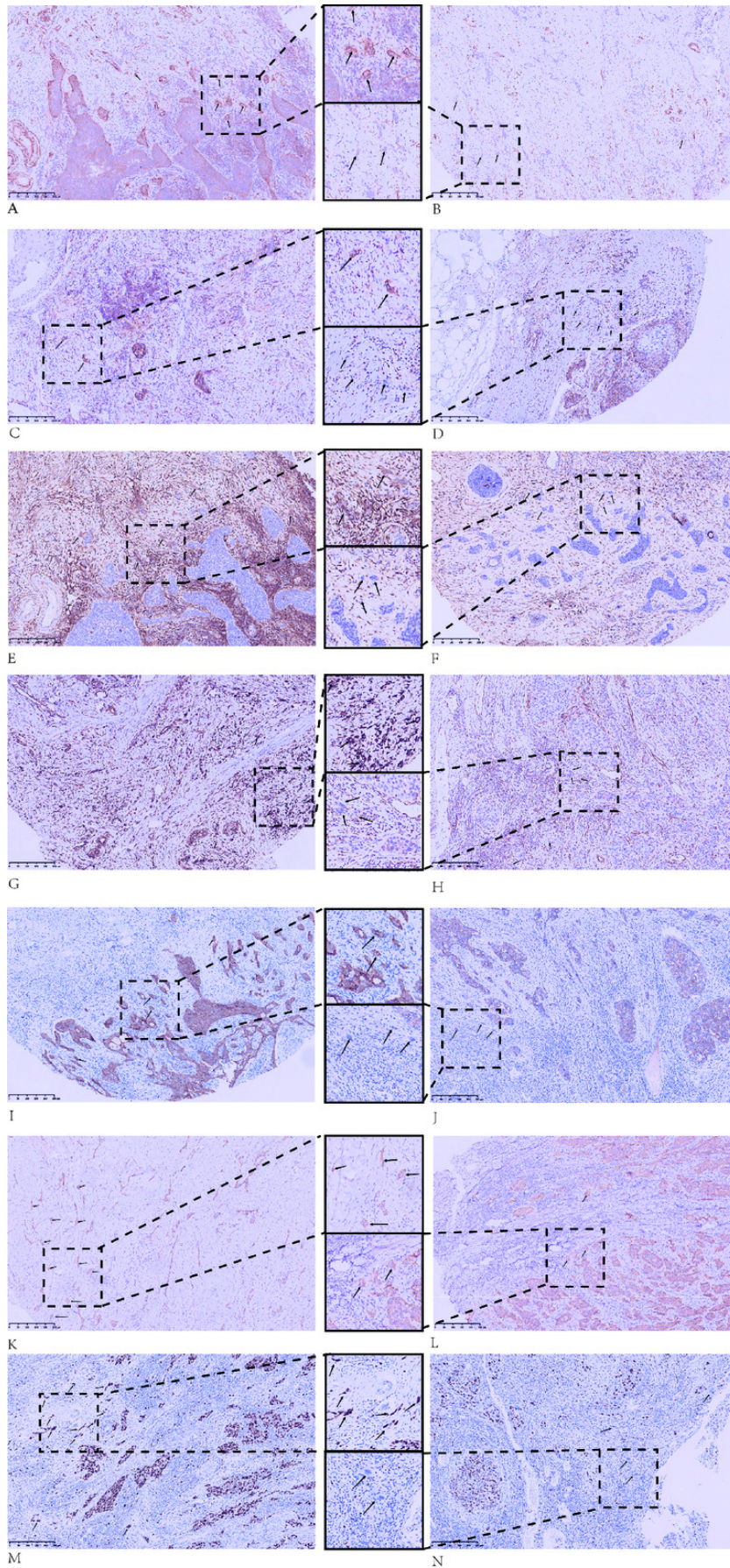
Figure 3

Fig. 3. Immunohistochemical (IHC) staining results for the main tumor body.

The main tumor body that is: (A) negative for SOX2; (B) positive for SOX2; (C) weakly positive for CD44; (D) strongly positive for CD44; (E) ALDH1A1 negative; (F) ALDH1A1 positive; (G) vimentin negative; (H) vimentin positive; (I) weakly positive for E-cadherin; (J) strongly positive for E-cadherin; (K) positive for cytoplasmic β-catenin; and (L) positive for membrane β-catenin.IHC staining of the main tumor body with Ki67 showing: (M) low proliferation and (N) high proliferation.All images: magnification, 100X

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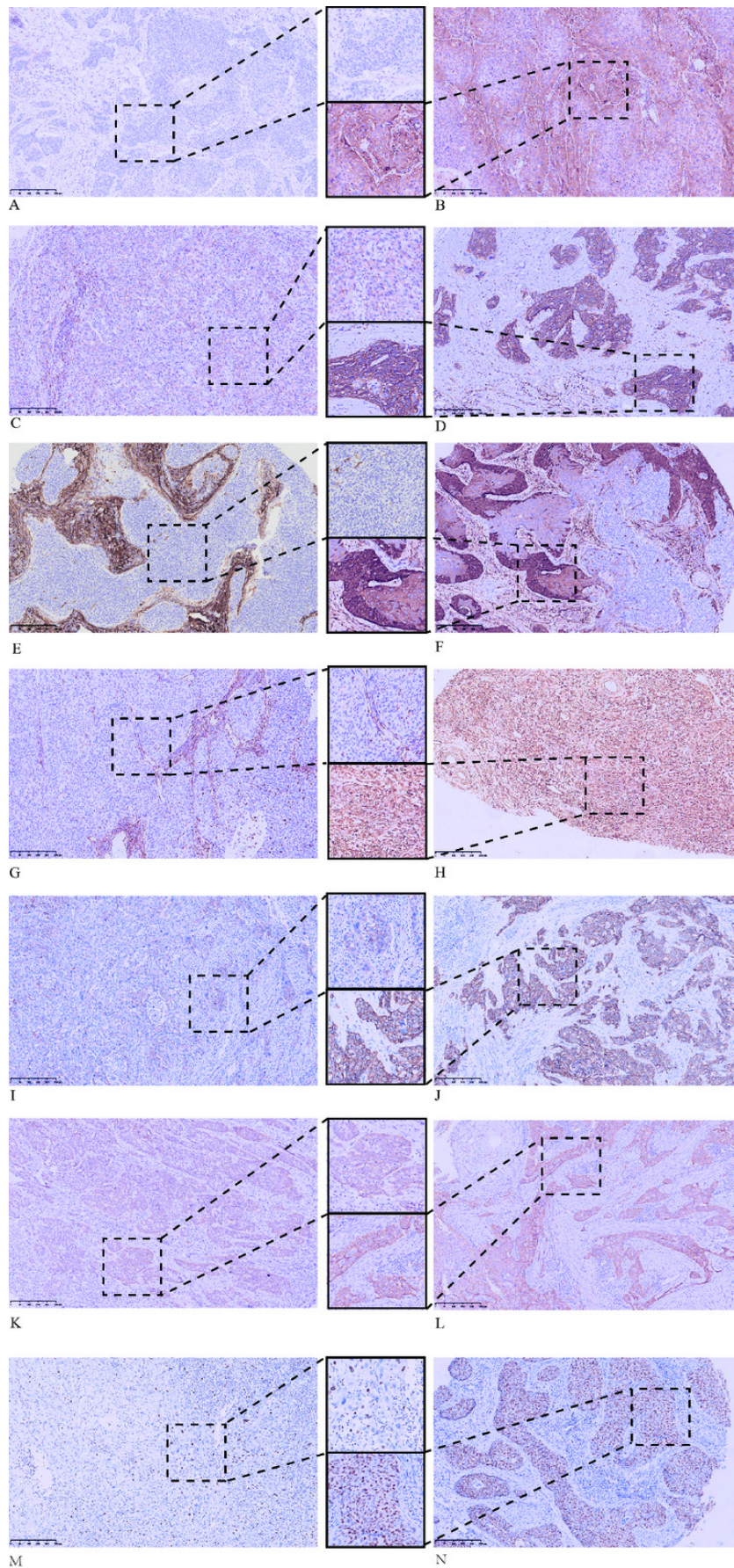
Table 1. Patient characteristics

1   **Table 1. Patient characteristics**

Clinical feature		No. of patients	Tumor budding		P-value[a]
			YES	*NO*	
Age (years)	Maximum	78			
	Minimum	26			
	Median	52			
	<50 y	41	39	2	0.477
	≥50 y	54	48	6	
Clinical stages	Phases Ib1, Ib2	43	39	4	1.000
	Phases IIa1, IIa2	52	48	4	
Lymphatic invasion	Yes	43	39	4	1.000
	No	52	48	4	
Lymphatic metastasis	Yes	12	11	1	1.000
	No	83	76	7	

2   P-value[a], *P*-value of the chi-squared tests

Table 2. Immunohistochemical staining of tissue microarrays

1   **Table 2. Immunohistochemical staining of tissue microarrays**

Marker	Group	Expression, n (%)		χ^2-value	P-value
		Negative-/Low	Moderate-/High		
SOX2	1	42 (48.3%)	45 (51.7%)	37.144	<0.001
	2	79 (90.8%)	8 (9.2%)		
CD44	1	75 (86.2%)	12 (13.8%)	20.221	<0.001
	2	48 (55.2%)	39 (44.8%)		
ALDH1A1	1	42 (48.3%)	45 (51.7%)	14.653	<0.001
	2	18 (20.7%)	69 (79.3%)		
ᵥVimentin	1	68 (78.2%)	19 (21.8%)	13.321	<0.001
	2	84 (96.6%)	3 (3.4%)		
E-cadherin	1	67 (77.0%)	20 (23.0%)	81.575	<0.001
	2	8 (9.2%)	79 (90.8%)		
Ki67	1	71 (81.6%)[a]	16 (18.4%)[b]	101.765	<0.001
	2	5 (5.7%)[a]	82 (94.3%)[b]		

2   Group 1, tumor budding; Group 2, main tumor body

3   [a]Ki67-expression, no reaction/weak reaction

4   [b]Ki67-expression, moderate reaction/intense reaction