The ratio of serum Angiopoietin-1 to Angiopoietin-2 in patients with cervical cancer is a valuable diagnostic and prognostic biomarker

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Objectives: Angiopoietins have found to play essential roles in tumor angiogenesis. The present study was aimed to investigate the diagnostic and prognostic values of serum angiopoietin 1 and 2 (sAng-1 and sAng-2) in cervical cancer. **Methods:** The sAng-1 and sAng-2 concentrations were analyzed in 77 women with cervical cancer, 44 women with cervical intraepithelial neoplasia (CIN) and 43 women without cervical lesions by enzyme-linked immunosorbent assay. The diagnostic values of sAng-1, sAng-2 and sAng-1/sAng-2 were evaluated by receiver operating characteristic (ROC) curves. The Ang-1 and Ang-2 expression, as well as microvessel density (MVD), in cervical cancer tissues were assessed by immunohistochemistry. **Results:** The concentration of sAng-2 gradually increased and the sAng-1/Ang-2 ratio was gradually decreased from normal control to CIN, then to squamous cell cancer, and the sAng-1/sAng-2 ratio was also significantly decreased in adenocarcinoma. The area under ROC curves of sAng-2 and sAng-1/sAng-2 ratio for discriminating cervical cancer from normal were 0.744 and 0.705, respectively. Decreased sAng-1/sAng-2 was significantly associated with advanced tumor stage, poor differentiation, lymph-vascular space invasion and high MVD. sAng-2 was positively correlated with the Ang-2 expression in cervix epithelia. A high sAng-1/sAng-2 ratio was associated with a longer progression-free survival and a longer overall survival in cervical cancer patients. **Conclusions:** These findings suggest that sAng-2 and the sAng-1/sAng-2 ratio may be valuable diagnostic and prognostic biomarkers for cervical cancer.

1 **The ratio of serum Angiopoietin-1 to Angiopoietin-2 in patients with cervical cancer is a**

2 **valuable diagnostic and prognostic biomarker**

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22 **Abbreviation**

23 angiopoietin, Ang; serum Ang-2, sAng-2; vascular endothelial growth factor, VEGF; cervical

24 intraepithelial neoplasia, CIN; microvessel density, MVD; cervical squamous cell carcinoma,

25 CSCC; cervical adenocarcinoma, CADC; lymphovascular space invasion, LVSI; Progression-

26 free survival, PFS; Overall survival, OS; enzyme-linked immunosorbent assay, ELISA;

27 Horseradish Peroxidase, HRP; immunohistochemistry, IHC; diaminobenzidine, DAB;

28 endothelial cells, EC; high power field, HP.

29

30

31 **Abstract**

32 **Objectives:** Angiopoietins have found to play essential roles in tumor angiogenesis. The present

33 study was aimed to investigate the diagnostic and prognostic values of serum angiopoietin 1 and

34 2 (sAng-1 and sAng-2) in cervical cancer. **Methods:** The sAng-1 and sAng-2 concentrations

35 were analyzed in 77 women with cervical cancer, 44 women with cervical intraepithelial

36 neoplasia (CIN) and 43 women without cervical lesions by enzyme-linked immunosorbent assay.

37 The diagnostic values of sAng-1, sAng-2 and sAng-1/sAng-2 were evaluated by ROC. In

38 addition, the Ang-1 and Ang-2 expression, as well as microvessel density (MVD), in cervical

39 cancer tissues were assessed by immunohistochemistry. **Results:** The concentration of sAng-2

40 gradually increased and the sAng-1/sAng-2 ratio was gradually decreased from normal control to

41 CIN, then to squamous cell cancer, and the sAng-1/sAng-2 ratio was also significantly decreased

42 in adenocarcinoma. The area under ROC curves of sAng-2 and sAng-1/sAng-2 ratio for

43 discriminating cervical cancer from normal were 0.744 and 0.705, respectively. Decreased sAng-

44 1/sAng-2 was significantly associated with advanced tumor stage, poor differentiation, lymph-

45 vascular space invasion and high MVD. sAng-2 was positively correlated with the Ang-2

46 expression in cervix epithelia. A high sAng-1/sAng-2 ratio was associated with a longer

47 progression-free survival and a longer overall survival in cervical cancer patients. **Conclusions:**

48 These findings suggest that sAng-2 and the sAng-1/sAng-2 ratio may be valuable diagnostic and

49 prognostic biomarkers for cervical cancer.

50

51 **1. Introduction**

52 Globally, cervical cancer incidence has increased from 378,000 cases per year in 1980 to

53 528,000 cases per year in 2012, representing a 0.6 % annual rate of increase (Ferlay et al. 2015).

54 The majority of cervical cancer patients come from the economically underdeveloped areas, and

55 they have higher mortality. Although the cervical screening programs based on the cervical

56 cytology and HPV test can effectively reduce the incidence of cervical cancer, women in

57 underdeveloped areas usually cannot accept standardized screening due to social, religious and

58 psychological factors. Thus, novel circulating biomarkers that allow monitoring of essential

59 molecular events in cervical cancer may improve the detection of lesions that have a high risk of

60 progression in both primary screening and triage settings.

61

62 Angiogenesis is essential for tumor progression and has shown great promise as a therapeutic

63 target for treatment of advanced cervical cancer (Eklund & Saharinen 2013; Kerbel 2015).

64 Recently, agents targeting the angiopoietin (Ang)/Tie system have been developed in several

65 clinical trials and achieved encouraging results in anti-angiogenesis therapy (Gerald et al. 2013).

66 The human Ang/Tie system is mainly composed of three secreted ligands (Ang-1, Ang-2, and

67 Ang-4) and two tyrosine kinase receptors Tie-1 and -2. All three human angiopoietins bind

68 directly to Tie-2 and result in phosphorylation and multimerization of Tie-2, which in turn

69 trigger downstream signaling and regulate vascular remodeling and maturation. Ang-1 functions

70 as a constitutive Tie-2 receptor agonist. The function of Ang-2 is context-dependent; Ang-2 functions

71 as an partial Tie-2 signaling agonist in the presence of Ang-1 or a Tie-2 signaling agonist in the

72 absence of Ang-1 (Augustin et al. 2009; Fagiani & Christofori 2013; Yang et al. 2015). Ang-1 is

73 widely expressed in periendothelial cells in human quiescent vasculature, where it sustains vessel

74 maturation and stabilization. In contrast, Ang-2 is expressed in endothelial cells during vascular

75 remodeling and angiogenesis, where it mediates vascular destabilization. Thus, the balance of

76 Ang-1 and Ang-2, as well as the cellular context of Tie-2 activation, contribute to the outcome of

77 Tie-2 activity. In addition, angiopoietins have been shown to directly interact with integrins on

78 endothelial, mesenchymal and tumor cells, thereby eliciting Tie-2-independent biological effects

79 (Augustin et al. 2009; Fagiani & Christofori 2013; Shim et al. 2007). Both Ang-1 and -2 have

80 recently been found to be expressed in tumor cells, and they act cooperatively with VEGF

81 promoting the angiogenesis during tumor progression (Augustin et al. 2009; Ebos & Kerbel 2011;

82 Gerald et al. 2013; Shim et al. 2007; Yang et al. 2015).

83

84 Increased circulating serum Ang-2 (sAng-2) concentrations or a shift in the sAng-1/sAng-2 ratio

85 in favor of sAng-2 in tumor patients has been shown to correlate with advanced tumor

86 progression and poor survival in epithelial ovarian cancer (Sallinen et al. 2014; Sallinen et al.

87 2010), melanoma (Helfrich et al. 2009), metastatic colorectal cancer (Goede et al. 2010),

88 neuroendocrine tumors (Detjen et al. 2010), hepatocellular carcinoma (Bouattour et al. 2015) and

89 pancreatic cancer (Schulz et al. 2011). Furthermore, a soluble Tie-2 receptor can sequester

90 angiopoietins (Augustin et al. 2009; Schulz et al. 2011). In cervical cancer, based on a

91 preliminary study on plasma concentrations of Ang-1and Ang-2 in 34 patients with cervical

92 cancer, circulating Ang-1, Ang-2, and Tie-2 and the Ang-1/Ang-2 ratio were significantly

93 increased compared with healthy women (Kopczynska et al. 2009). Moreover, decreased Ang-

94 1/Ang-2 ratio was associated with advanced Federation of Gynecology and Obstetrics (FIGO)

95 stage (Kopczynska et al. 2009). However, the diagnostic and predictive value of circulating Ang-

96 1 and Ang-2 in cervical cancer remains largely unclear.

97

98 In the present study, the diagnosis and prognostic value of sAng-1, -2 and sAng-1/sAng-2 in

99 patients with cervical cancer was evaluated. In addition, the correlations between the circulating

100 angiopoietin concentrations and the angiopoietin expression levels and microvessel density

101 (MVD) in tumor were investigated.

102

103 **2.   Materials and methods**

104 **2.1 Study groups and inclusion criteria**

105 We conducted a non-matched case-control study using serum samples obtained from patients

106 who received treatment at Wuhan Union Hospital (Wuhan, Hubei, China) between February

107 2012 to March 2014, including 44 patients with CIN, 77 patients with cervical cancer (61

108 squamous cell carcinomas, CSCC, and 16 adenocarcinoma, CADC) and 43 patients with benign

109 disorders of uterus such as myoma or adenomyosis as controls. The diagnoses were

110 pathologically verified and patients with medical diseases such as other type of cancer or

111 inflammatory, atherosclerotic and connective tissue disease were excluded. The age range of the

112 164 patients was 23-72 years with mean of 44.26 years, and there was no significant difference

113 in age among the cervical cancer, CIN and control groups. All patients or their next of kin

114 provided written informed consent for the collection of samples and subsequent research. The

115 present study was approved by the Ethics Committee of Tongji Medical College, Huazhong

116 University of Science and Technology (IORG0003571).

117

118 **2.2 Clinical definitions**

119 The medical records of the patients with cervical cancer were reviewed to collect clinical and

120 pathological characters, including age at diagnosis, FIGO stage, pathological type, tumor

121 differentiation, pelvic lymph node metastasis, tumor size, and lymphovascular space invasion

122 (LVSI). The patients were classified according to the revised FIGO staging system for cervical

123 cancer of 2009. The tumor size was the largest diameter of tumor determined by the attending

124 gynecologic oncologist during a pelvic examination preceding surgery. The patients of IA1 stage

125 underwent hysterectomy, and patients between IB1 and IIB underwent radical hysterectomy and

126 pelvic lymph node dissection. Patients with ≥IIB stage underwent radiotherapy or radiotherapy

127 combined with chemotherapy. Stage-specific follow-up after treatment was performed in

128 accordance with the FIGO guidelines. Progression-free survival (PFS) was defined as the time

129 from treatment to the first appearance of tumor recurrence or to the date of last contact. Overall

130 survival (OS) was defined as the time from treatment to death from any cause or to the date of

131 last contact.

132

133 **2.3 Sample collection, ELISA, and IHC**

134 Serum samples were collected before primary treatment such as surgery, radiation, or

135 chemotherapy. Blood was drawn into serum tubes (10 ml) and centrifuged at 2200 G/min for 10

136 minutes. Serum was harvested, aliquoted and stored at −80°C until usage.

137

138 The concentration of sAng-1 and sAng-2 in serum was assayed by a standardized sandwich

139 enzyme-linked immunosorbent assay (ELISA) in triplicate according to the protocol

140 recommended by the manufacturer (Uscn Life Science Inc. Wuhan, China).

141

142 For immunohistochemistry (IHC), paraffin-embedded sections (4μm) were hydrated by alcohol

143 and then subjected to antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). After being

144 treated with 3% H_2O_2 for 10 minutes, samples were incubated with primary antibodies overnight

145 at 4 °C. Three primary antibodies were used: human antibodies for detection of Ang-1(AF923,

146 Polyclonal, Goat IgG), Ang-2 (AF623, Polyclonal, Goat IgG) and CD34 (AF7227, Polyclonal,

147 Sheep IgG) in cervical tissue were all purchased from R&D (Minneapolis, MN, USA); the

148 working concentrations of antibodies were 5 μg/ml. Standard horseradish peroxidase staining

149 using an appropriate biotinylated secondary antibody (ZSGB-BIO, Peking, PRC), the elite ABC

150 kit (Avidin: Biotinylated enzyme Complex; ZSGB-BIO, Peking, PRC) and diaminobenzidine

151 (ZSGB-BIO, Peking, PRC) were performed according to the manufacturers' instructions.

152 Sections were counterstained with Gill's haematoxylin (Sigma, Aldrich, St. Louis, MO USA).

153 The expression of angiopoietins in epithelial cells and endothelial cells (EC) in cervical tissues

154 were separately evaluated and scored as negative staining and positive staining. MVD was

155 assessed using CD34 (Yang et al. 2009).

156

157 **2.4 Statistical analysis**

158 Statistical analysis was carried out with SPSS 13.0 statistical software (SPSS, Chicago, IL, USA).

159 Continuous variables were expressed as median and range; the data from each category was

160 presented as frequency and percentage. Non-parametric tests were used for comparisons between

161 different groups. The differences in sAng-1, sAng-2, and sAng-1/sAng-2 ratio between groups

162   were analyzed with the Kruskal-Wallis test combined with Dunn's multiple comparison, the

163   Jonckheere-Terpstra test or Mann-Whitney U test, as appropriate. ROC curve was used to

164   analyze the diagnostic value of individual factors. Correlations were analyzed with the

165   Spearman's correlation coefficient. Survival analyses were performed and Kaplan-Meier survival

166   curves were generated; the survival distributions were compared by log-rank test. All tests were

167   two-tailed, and a P value of less than 0.05 was considered to be statistically significant.

168

169   **3.   RESULTS**

170   **3.1 sAng-2 concentration and sAng-1/sAng-2 ratio are altered in patients with cervical**

171   **neoplasia**

172   The sAng-1and sAng-2 in 43 patients of normal control, 44 CIN patients and 77 cervical cancer

173   patients (61 CSCC patients and 16 CADC patients) were detected by ELISA. Given that CINs

174   are the precancerous lesions of CSCCs, we first compared the sAng-1, sAng-2 and sAng-1/sAng-

175   2 ratio in the normal, CIN and CSCC patients. We found that the sAng-1 concentration was not

176   significantly different between these three groups, with a median concentration of 58.13 pg/ml

177   (range, 6.79-194.1 pg/ml) in the control group, 28.53 pg/ml (range, 7.22-248.5 pg/ml) in the CIN

178   group and 37.79 pg/ml (range, 1.57-361.2 pg/ml) in the CSCC group (Kruskal-Wallis test,

179   P=0.068, Fig. 1A). However, there was a significant difference in sAng-2 between the three

180   groups (Kruskal-Wallis test, P=0.000), and from normal control (28.87(1.86-235.7) pg/ml), to

181   CIN (44.35(0.70-395.6) pg/ml), then to CSCC (109.90(1.09-431.70) pg/ml), in that with the

182   increasing severity of cervical lesions, the concentration of sAng-2 gradually increased

183   (Jonckheere-Terpstra test, P <0.001; Fig. 1B). The sAng-1/sAng-2 ratio was also significantly

184   different between the three groups (Kruskal-Wallis test, P <0.001; Fig. 1C). The sAng-1/sAng-2

185   ratio decreased gradually from the control group (median, 2.07; range, 0.25-30.36), to the CIN

186   group (median, 0.85; range, 0.08-17.54), then to the CSCC group (median,0.54; range, 0.02-

187   42.91) (Jonckheere-Terpstra test, P <0.001; Fig. 1C).

188

189 We then compared the concentration of sAng-1and sAng-2 and the sAng-1/sAng-2 ratio in the

190 CADC group relative to the normal groups. The differences in sAng-1(37.79 vs 58.13 pg/ml) and

191 sAng-2 concentrations (62.97 vs 28.87 pg/m) were not significant (Fig. 1A, B). However, the

192 sAng-1/sAng-2 ratio was significantly decreased in CADC patients compared with the normal

193 group (0.58(0.06-33.33) vs 2.07(0.25-30.36), P=0.008) (Fig. 1C).

194

195 **3.2 sAng-2 concentration and the sAng-1/sAng-2 ratio are valuable diagnostic biomarkers**

196 **for cervical lesions**

197 Having determined the differential sAng-2 concentrations and sAng-1/sAng-2 ratio in cervical

198 cancer lesions compared with normal, we then used ROC curves to test whether the sAng-2

199 concentration and sAng-1/sAng-2 ratio can serve as biomarkers to distinguish CSCC from

200 normal and CIN. The data of the 43 patients of normal control, the 44 CIN patients, and the 61

201 CSCC patients were included in the analysis. The area under curve (AUC) of the ROC curves of

202 sAng-2 for discriminating CSCC from normal (Fig. 2A), CSCC from CIN (Fig. 2B), CSCC from

203 normal and CIN (Fig. 2C), CIN and CSCC from normal (Fig. 2D), and invasive cervical cancer

204 from normal (Fig. 2E) were 0.769, 0.662, 0.715, 0.706 and 0.744, respectively. The sAng-2

205 cutoff value of 48.24 pg/ml provides a high diagnostic accuracy to discriminate CSCC from

206 normal, with a sensitivity of 76.67% and a specificity of 74.42% (Fig. 2F).

207

208 The AUC value of sAng-1/ sAng-2 as a diagnostic biomarker for normal to CIN, normal to

209 CSCC, normal to CIN and CSCC and normal to cervical cancer was 0.657, 0.719, 0.693 and

210 0.705 (Fig. 2G-J), respectively. The cutoff value of 1.09 had a sensitivity of 68.83% and

211 specificity of 72.09% to distinguish CSCC from normal (Fig. 2K).

212

213 **3.3 sAng-1 and sAng-2 correlate to clinicopathological characteristics in patients with**

214 **cervical cancer**

215 The correlations between the concentrations of serum angiopoietins and clinicopathological

216   characteristics of cervical cancer were analyzed in a subset of 77 patients with cervical cancer,

217   including 41 stage IA1 to IB1, 20 stage IB2 to IIA and 16 stage ≥IIB based on classification

218   criteria (FIGO, 2009). Characteristics of the patients are summarized in Table 1. We found that

219   sAng-1 concentration was significantly decreased in groups of stage ≥IB2, tumor size > 2 cm and

220   LVSI compared with stages IA1-IB1, tumor size ≤ 2 cm, or no LVSI, respectively. sAng-1

221   concentrations had no significant correlation with differentiation and pelvic lymphatic status.

222   Although the sAng-2 concentration was significantly increased in the poorly differentiated group,

223   no significant correlation with other clinicopathological characteristics were noted. sAng-

224   1/sAng-2 was significantly decreased in stages ≥IB2, poor differentiation and LVSI, but had no

225   significant correlation with pelvic lymphatic metastasis status and tumor size (Table 2).

226

227   **3.4 sAng-2 concentration positively correlates with Ang-2 expression on epithelia and MVD**

228   **in cervical tissues**

229   In a subset of 25 patients with invasive cervical cancer (FIGO stage IA2-IIA) and 10 normal

230   control patients, angiopoietin expression and MVD were evaluated in paraffin-embedded

231   cervical tissue samples. Among the 25 cervical cancers, 21 cases were squamous cancer and 4

232   were adenocarcinoma; 15 patients had a tumor stage of FIGO IA1 to IB1 and 10 had a tumor

233   stage of FIGO IB2 to IIA; 7 tumors were well differentiated (G1), 8 were moderately

234   differentiated (G2) and 10 were poorly differentiated cancer (G3); 9 patients had lymphovascular

235   space invasion (LVSI); 4 patients had pelvic lymph node metastasis; the tumor sizes were greater

236   than 2 cm in 9 cases.

237

238   The IHC assays revealed that both Ang-1 and Ang-2 were highly expressed on tumor cells

239   compared to normal cervical epithelia. The positive expression rate of Ang-1 in cervical cancer

240   epithelia was 60% (15/25) and Ang-2 was 68% (17/25), while normal cervical epithelia had no

241   positive staining. For the expression of Ang-1 and Ang-2 in endothelial cells, there was no

242   significant difference between cervical cancer tissue specimens and normal controls (Fig. 3A, B).

243

244   MVD, as assessed by CD34 staining, was significantly increased in cervical cancer compared

245   with normal (Fig. 3C). The patients with positive Ang-2 in cervical epithelia had significantly

246   elevated sAng-2 concentration when compared to those with negative Ang-2 expression in

247   cervical epithelia (80.89 (15.54-431.7) pg/ml vs 24.66 (1.86-299.3) pg/ml) (P= 0.029, Fig. 3D).

248   In addition, the sAng-1 concentration did not correlate with MVD (r=0.256, P= 0.138, Fig. 3E);

249   the sAng-2 concentration positively correlated to MVD (r=0.440, P= 0.008, Fig. 3F); the sAng-

250   1/sAng-2 ratio was negatively correlated to MVD (r=-0.516, P= 0.002, Fig. 3G).

251

252   **3.5 High sAng-1/sAng-2 ratio predicts poorer prognosis in cervical cancer patients**

253   The prognostic value of sAng-1, -2 and sAng-1/sAng-2 was evaluated in 66 cervical cancer

254   patients with follow-up data. The patients were followed up to October 2016. The median

255   follow-up time was 33.62 months (range, 9-48 months). Of the 66 patients, 17 suffered from

256   recurrence and 8 died. According to the ROC analyses, we choose 131.00 pg/ml, 156.40 pg/ml,

257   and 0.26 as the cut-off values of sAng-1, sAng-2 and sAng-1/sAng-2, respectively, as these

258   values were associated with a specificity of 95% for discriminating between invasive cervical

259   cancer and normal. We found that there were no significant differences in the PFS and OS

260   between the patients with high versus low sAng-1 or sAng-2 (Fig. 4A-D). However, the sAng-

261   1/sAng-2 ratio was significantly associated with PFS (Fig. 4E, P=0.046) and OS (Fig. 4F,

262   P=0.040) in cervical cancer patients. In the low sAng-1/sAng-2 ratio group, 7 of 14 (50.0%)

263   patients developed recurrent disease and 4 (28.6%) patients died. The estimated 3-year PFS and

264   OS were 42.86% and 71.43%, respectively. In the high sAng-1/sAng-2 ratio group, 10 of 52

265   (19.23%) patients had recurrence and 4 (7.7%) patients died, resulting in an estimated 3-year

266   PFS and OS of 78.96% and 91.55%, respectively.

267

268   **4. Discussion**

269   In the present study, we found that the concentration of sAng-2 was significantly increased in

270 patients with cervical cancer compared with normal controls and CIN patients and the sAng-

271 1/sAng-2 ratio was decreasing during the malignant transformation of cervical epithelia.

272 Moreover, the sAng-2 and the sAng-1/sAng-2 ratio exhibit encouraging accuracies for diagnosis

273 of a CSCC. In patients with cervical cancer, low sAng-1/sAng-2 ratio was significantly

274 associated with advanced stage, poor differentiation, LVSI, high MVD and poor survival. These

275 findings suggest that the ratio of serum Angiopoietin-2 to Angiopoietin-1 is a valuable diagnostic

276 and prognostic biomarker in patients with cervical cancer.

277

278 Tumor angiogenesis is the most fundamental trait for tumor growth. Tumor nodules cannot

279 derive nutrients through diffusion when greater than 1–2 mm^3 (Hanahan & Weinberg 2011; Jain

280 & Carmeliet 2012). Previous reports on various type of cancer, such as colorectal cancer, gastric

281 carcinoma, and glioma, have shown that elevated Ang-2 in these tumor cells resulted in

282 increased MVD and stimulated tumor angiogenesis (Augustin et al. 2009; Fagiani & Christofori

283 2013; Shim et al. 2007). Moreover, in melanoma, tumor cells secreted sAng-2 and expressed

284 Tie-2 suggesting that Ang-2 may act as an autocrine regulator of tumor cell migration and

285 invasion (Helfrich et al. 2009). We found that Ang-2 was expressed in tumor cells of cervical

286 cancer tissues. The sAng-2 concentration also positively correlated with Ang-2 expression in

287 epithelia and MVD of cervical tissues, suggesting that the concentration of sAng-2 may reflect

288 the activity of blood vessel formation and soluble Ang-2 secreted by cervical cancer cells may be

289 the main source of serum sAng-2 of cervical cancer. Given that the sAng-2 and the sAng-

290 1/sAng-2 ratio were significantly associated with MVD in cervical cancer (Fig. 3F-G), they

291 might be used to evaluate the response of tumor to anti-angiogenesis therapy in real-time. In

292 metastatic colorectal cancer, sAng-2 was a candidate biomarker for outcome of patients treated

293 with bevacizumab-containing therapy (Goede et al. 2010). Very recently, a study indicated

294 bevacizumab reduced mortality by 30% in patients with recurrent, persistent, or metastatic

295 cervical cancer when combined with chemotherapy compared with those who receive

296 chemotherapy alone (Penson et al. 2015). The utility of sAng-2 and sAng-1/sAng-2 ratio as

297 biomarkers to predict outcome of bevacizumab-containing treatment in advanced cervical cancer

298 should be further validated.

299

300 We found that sAng-2 concentration gradually increased and the ratio of sAng-1/ sAng-2

301 gradually decreased with the severity of cervical lesion from control to CIN to CSCC. The sAng-

302 1/sAng-2 ratio was also decreased in CADC patients compared with normal group. In a previous

303 study, Kopczynska et al. assessed plasma concentrations of Ang-1and Ang-2 in 34 patients with

304 cervical cancer and 20 healthy volunteers. They reported that plasma concentrations of Ang-1

305 and Ang-2 were significantly higher in cervical cancer patients than in controls. Moreover, the

306 plasma Ang-1/Ang-2 ratios was increased in cervical cancer compared with control and was

307 higher in stage I than in stage II-III (Kopczynska et al. 2009). The increase in circulating Ang-2

308 in patients with cervical cancer was confirmed by our findings. However, the change in Ang-

309 1/Ang-2 ratio reported by Kopczynska et al. was opposite to that shown in our study and we did

310 not find significant differences in Ang-1 concentration between control, CIN, and cervical cancer.

311 These inconsistencies may be related to the differences in study population and the materials

312 used in ELISA. In addition, the influence of anticoagulants used during blood collection on

313 plasma angiopoietin detection cannot be excluded.

314

315 The ability of sAng-2 concentration and the ratio of sAng-1 to sAng-2 to distinguish between

316 normal, CIN, and cervical cancer was similar to results reported in epithelial ovarian cancer,

317 where the area under the curve for serum Ang-2 in ROC analysis was 0.75 to differentiate

318 ovarian cancer from benign or borderline ovarian tumors (Sallinen et al. 2014; Sallinen et al.

319 2010). In melanoma, use of 90% quantile of healthy controls as a cutoff value could discriminate

320 well between control individuals and individuals with melanoma stage III or IV patients

321 (Helfrich et al. 2009). sAng-2 was elevated in patients with hepatocellular carcinoma, suggesting

322 the potential use of angiopoietin-2 as a valuable diagnostic biomarker for the detection of

323 hepatocellular carcinoma (Bouattour et al. 2015). Our findings suggest that sAng-2 concentration

324   and the ratio of sAng-1 to sAng-2 may be novel diagnostic biomarkers for the detection of

325   normal, CIN and cervical cancer. Additional research to determine cutoff values based on

326   population characteristics are being explored.

327

328   Both sAng-1 and sAng-2 have been found to be involved in tumor growth and progression. In

329   most conditions, elevated sAng-1 played anti-tumorigenic effects, whereas upregulated sAng-2

330   stimulated tumor growth and progression (Augustin et al. 2009; Eklund & Saharinen 2013;

331   Fagiani & Christofori 2013; Shim et al. 2007). In some tumors, such as glioblastoma, breast

332   cancer, hepatocellular carcinoma and pancreatic cancer, both sAng-1 and sAng-2 are upregulated

333   (Augustin et al. 2009; Fagiani & Christofori 2013; Shim et al. 2007; Eklund & Saharinen 2013).

334   Elevated sAng-1 was found in lung cancer and epithelial ovarian cancer, yet no relation was

335   found with diagnosis and prognosis of tumor (Park et al. 2009; Sallinen et al. 2014). On the

336   contrary, patient with papillary thyroid cancer were found to have significantly decreased sAng-1

337   compared with the normal control (Makki et al. 2013). We found that sAng-2, but not sAng-1

338   was increased, suggesting that factors driving tumor angiogenesis may be unique to tumor types.

339

340   Elevated sAng-2 tightly correlated with lymphatic and liver metastasis and shorter survival in

341   pancreatic ductal adenocarcinoma (Schulz et al. 2011) and neuroendocrine tumor patients

342   (Detjen et al. 2010; Helfrich et al. 2009). sAng-2 has also been suggested to be a useful

343   biomarker in melanoma as progression and metastasis correlated with tumor load and overall

344   survival (Helfrich et al. 2009). In epithelial ovarian cancer, the serum concentration of Ang-2

345   predicted poor overall survival (Sallinen et al. 2014; Sallinen et al. 2010). In our study, we found

346   that lower sAng-1 in patients with cervical cancer correlated with more advanced cervical cancer,

347   including higher stage, larger tumor size and LVSI. We also found higher sAng-2 levels in

348   patients with poorly differentiated cervical cancer. Overall, the ratio of sAng-1/sAng-2 was

349   predictive of PFS and OS, showing that patient with cervical cancer and a lower sAng-1/sAng-2

350   had a poorer prognosis.

351

352 The present study assessed for the first time the serum concentrations of angiopoietins and sAng-

353 1/sAng-2 ratio in healthy women and patients with CIN and invasive cervical cancer and

354 evaluated the diagnostic and prognostic power of sAng-1, sAng-2 and sAng-1/sAng-2 in cervical

355 cancer. A major limitation of this study is the small sample size of cervical cancer (N=77),

356 particularly that of CADC (N=16), which may be associated with selection bias and low

357 statistical power. In addition, the follow-up periods of the patients were relatively short.

358 Multicenter studies with long-term follow-up are needed to verify our findings.

359

360 **5. Conclusion**

361 In summary, sAng-2 was significantly increased in cervical cancer patients and a low sAng-

362 1/sAng-2 ratio was highly effective of discriminating cervical cancer patients from normal

363 control and predictive of poor survival in patients with cervical cancer. These findings suggest

364 that the sAng-1/sAng-2 ratio is a potential diagnostic and prognostic biomarker for cervical

365 cancer. Further studies to validate these findings and explore the utility of anti-angiogenesis

366 therapeutic targets for treatment of advanced or recurrent cervical cancer are needed.

367

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Figure 1

sAng-2 concentration and sAng-1/sAng-2 ratio are altered in patients with cervical neoplasia

sAng-1 and sAng-2 concentrations in 43 patients of normal control, 44 CIN patients, and 77 cervical cancer patients (62 CSCC patients and 15 CADC patients) were determined by ELISA. The differences in Ang-1 **(A)**, sAng-2 **(B)**, and the sAng-1/sAng-2 ratio **(C)** between groups are shown.

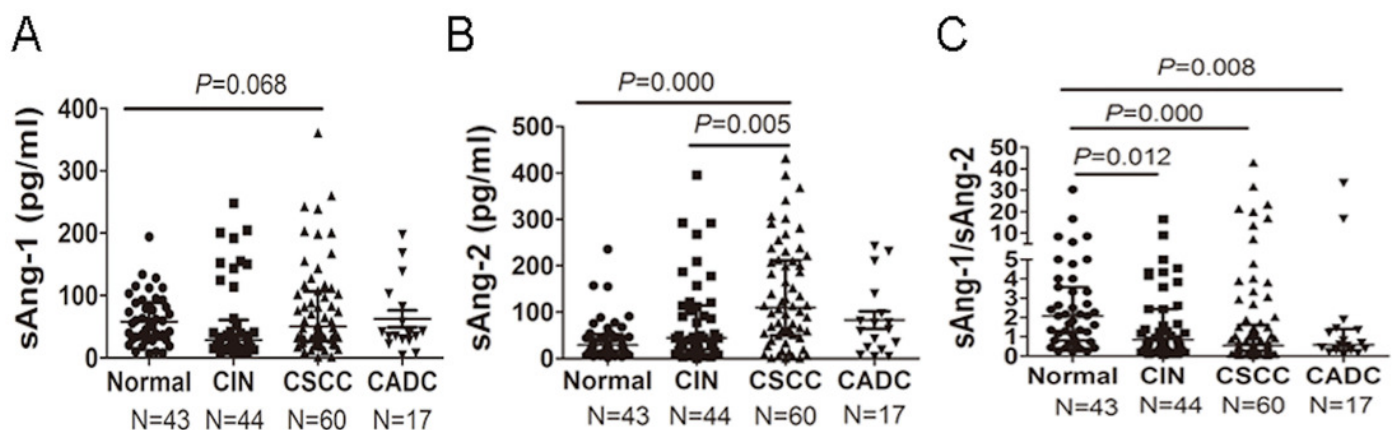



Figure 2

sAng-2 concentration and the sAng-1/sAng-2 ratio are valuable diagnostic biomarkers for cervical lesions

The ROC curves of sAng-2 for discriminating CSCC from normal **(A)**, CSCC from CIN **(B)**, CSCC from normal and CIN **(C)**, CIN and CSCC from normal **(D)**, and invasive cervical cancer from normal **(E)**. The cutoff values of sAng-2 and corresponding sensitivity and specificity to distinguish CSCC from normal are shown **(F)**. The ROC curves of sAng-1/ sAng-2 for discriminating CIN from normal **(G)**, CSCC from normal **(H)**, CIN and CSCC from normal **(I)** and invasive cervical cancer from normal **(J)**. The cutoff values of sAng-1/ sAng-2 and corresponding sensitivity and specificity to distinguish CSCC from normal are listed **(K)**.

F

variables	sAng-2 for normal to CSCC						
Sensitivity%	95.00	85.00	80.00	**76.67**	65.00	58.33	36.67
Specificity%	4.65	32.56	60.47	**74.42**	81.40	90.70	95.35
Cut-off value	4.30	19.26	38.48	**48.24**	66.00	89.23	156.2

K

variables	sAng-1/sAng-2 for normal to CSCC						
Sensitivity%	22.08	35.06	50.65	**68.83**	75.32	85.71	94.81
Specificity%	95.36	90.70	81.40	**72.09**	53.49	23.26	2.32
Cut-off value	0.26	0.39	0.58	**1.09**	1.70	3.80	20.70

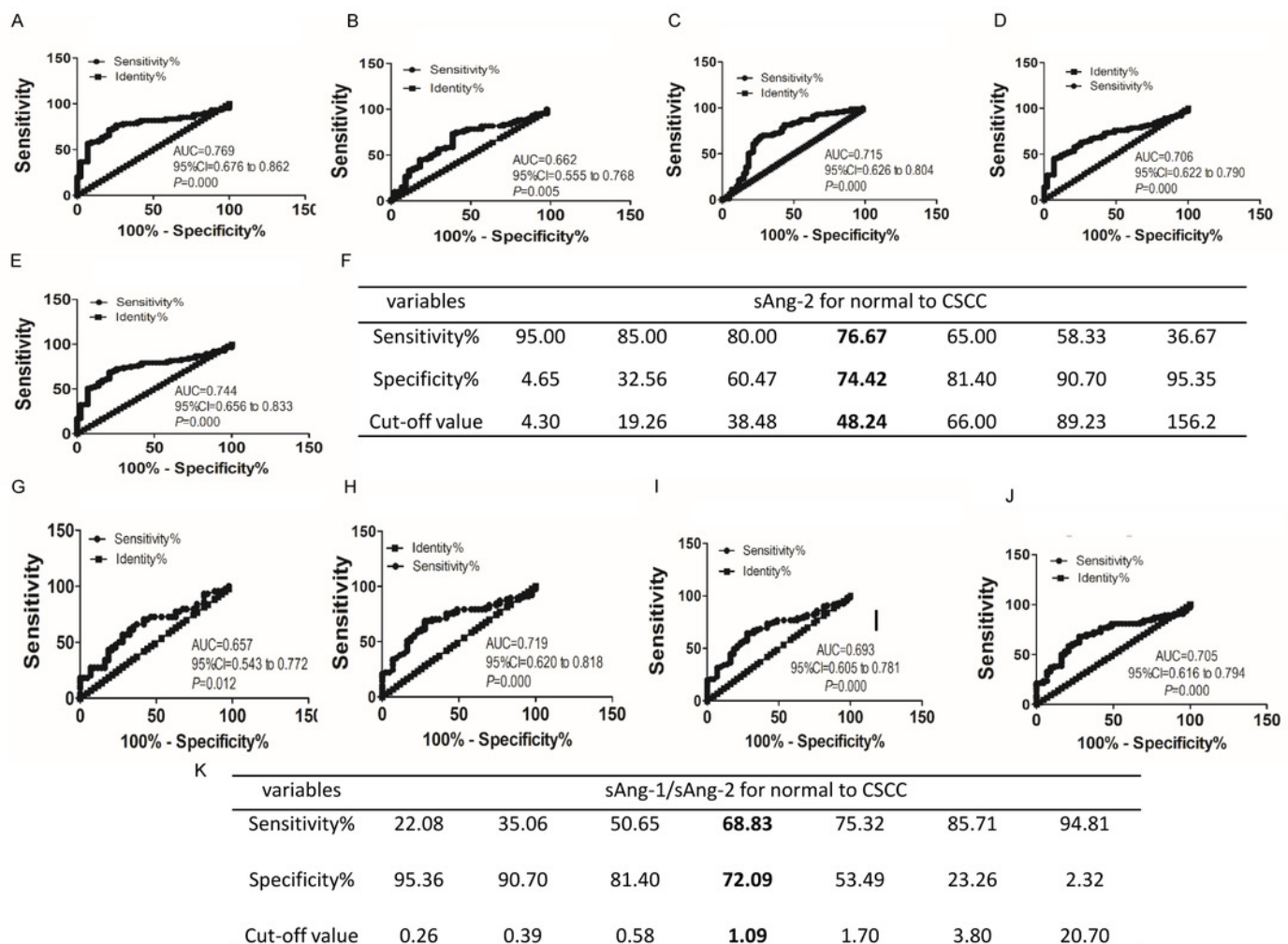



Figure 3

sAng-2 concentration positively relates with Ang-2 expression on the epithelia and MVD in cervical tissues

Representative immunohistochemical staining of Ang-1(**A**), Ang-2 (**B**) and CD34 **(C)** in 25 cervical cancer tissue specimens and 10 normal controls. Black arrows denote positively stained epithelial cells, whereas red arrows denote positively staining endothelial cells, all appearing brown. Scale bar, 20 μm. **(D)** sAng-2 is significantly higher in the patients with positive Ang-2 expression on cervix epithelia than those with negative Ang-2 expression. The scatter diagrams show the correlations of sAng-1 **(E)**, sAng-2 **(F)** and sAng-1/ sAng-2 ratio **(G)** to MVD in the 35 cervical tissue specimens.

![Figure 3 panels A–G showing immunohistochemical staining and scatter plots]

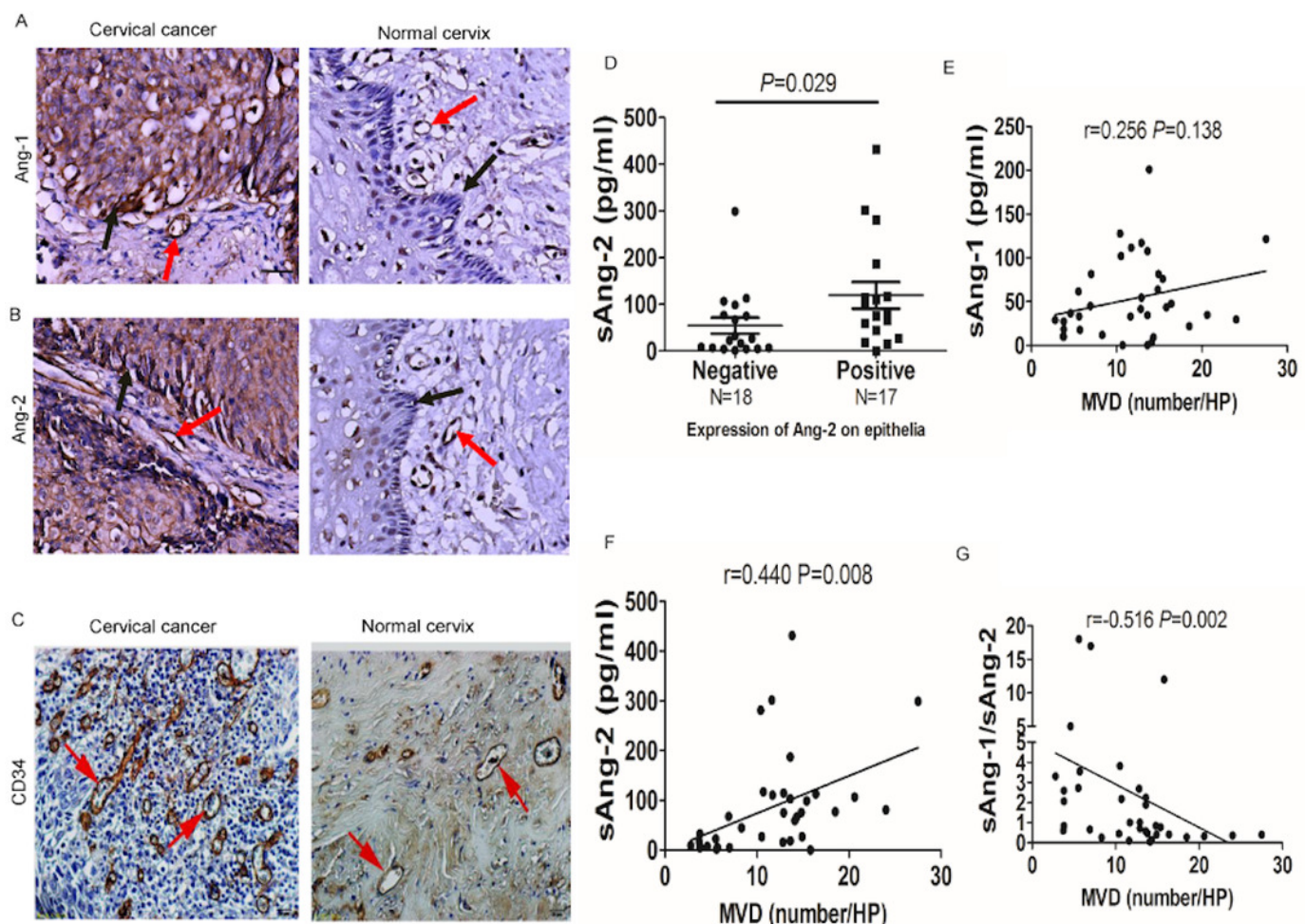
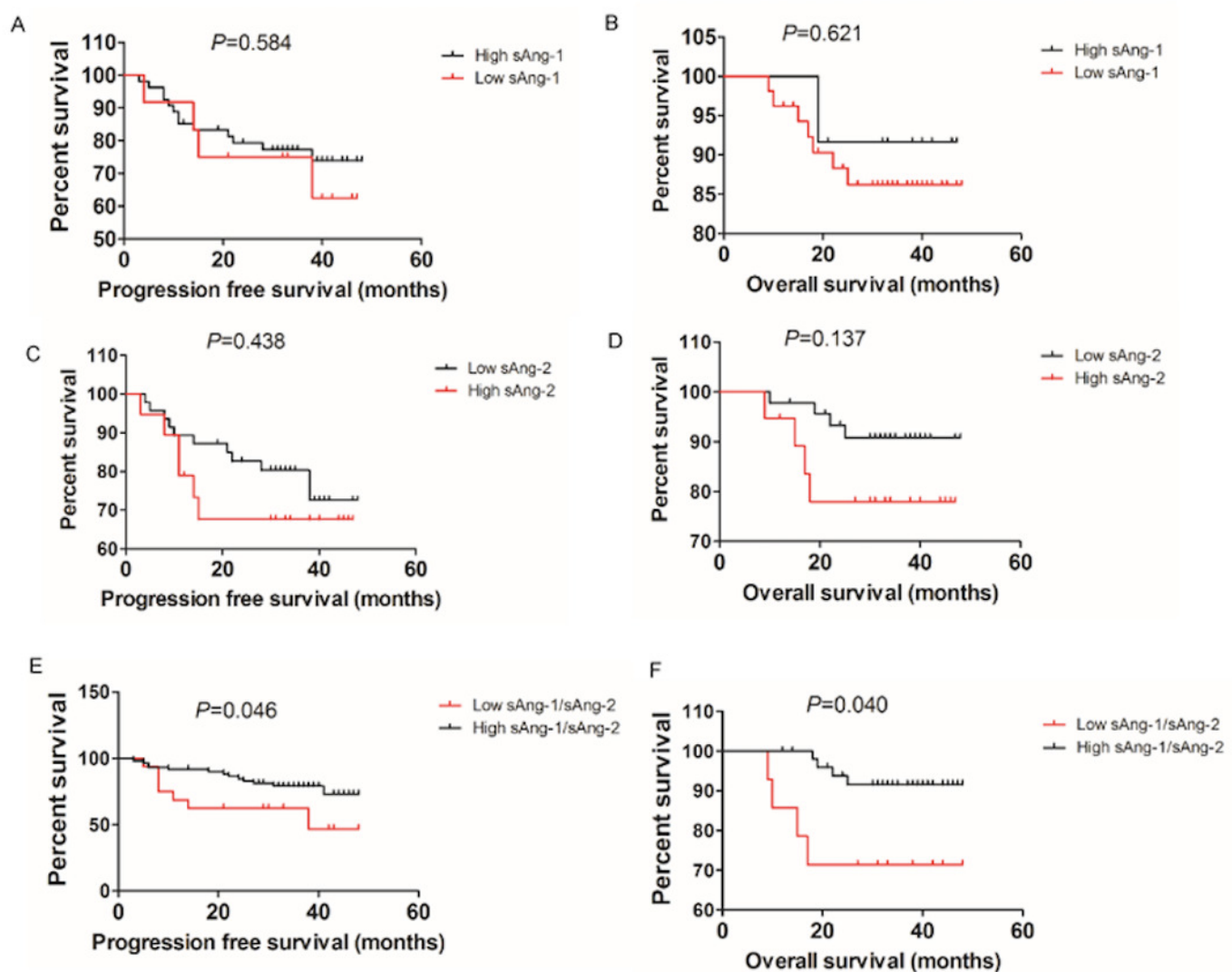



Figure 4

High sAng-1/sAng-2 ratio predicts poorer survival in cervical cancer patients

Kaplan-Meier survival analysis of the progression-free survival and the overall survival among 66 cervical cancer patients stratified by sAng-1**(A, B)**, sAng-2 **(C, D)** and sAng-1/sAng-2 ratio **(E, F)**.

The characteristics and serum angiopoietins in patients with cervical canc er (*N*=77).

1   Table 1 The characteristics and serum angiopoietins in patients with cervical cancer (*N*=77).

Parameters	Cases (%)
Age (year)	
Median (range)	46 (28-68)
≤ 45	37 (48.1)
> 45	40 (51.9)
FIGO stage	
IA1-IA2	3 (3.9)
IB1	38 (49.4)
IB2-IIA2	20 (25.9)
IIB	12 (15.6)
IIIA- IV	4 (5.20)
Pathological type	
Squamous carcinoma	61 (79.2)
Adenocarcinoma	16 (20.8)
Differentiation	
Well differentiated	19 (24.7)
Moderately differentiated	31 (40.2)
Poorly differentiated	27 (35.1)
Pelvic lymph node metastasis	
Negative	40 (51.9)
Positive	21 (27.3)
Unknown	16 (20.8)
Tumor size	
≤ 2cm	52 (67.5)
> 2cm	25 (32.5)
Lymphovascular space invasion	
Negative	48 (62.3)
Positive	29 (37.7)
sAng-1 (pg/ml)	
Median (range)	47.81 (1.57-361.21)
High	14 (18.2)
Low	63 (81.8)
sAng-2 (pg/ml)	
Median (range)	89.94 (1.09-431.68)
High	24 (31.2)
Low	53 (68.8)
sAng-1/sAng-2	
Median (range)	0.54 (0.02-42.91)
High	60 (77.9)

	Low	17 (22.1)

The correlation between sAng-1, sAng-2, sAng-1/sAng-2 ratio and clinicopathological characteristics in cervical cancer (*N*=77).

1 Table 2 The correlation between sAng-1, sAng-2, sAng-1/sAng-2 ratio and clinicopathological characteristics in cervical cancer

Variable	Cases	sAng-1 (pg/ml) Median (range)	P	sAng-2 (pg/ml) Median (range)	P	sAng-1/sAng-2 Median (range)	P
Age (year)							
≤ 45	37	59.92 (1.57-361.21)	0.266	66.28 (1.09-431.68)	0.203	0.77 (0.02-33.33)	0.101
> 45	40	37.96 (11.84-253.59)		113.70 (1.68-368.08)		0.46 (0.08-50.00)	
FIGO stage							
IA1-IB1	41	59.92 (1.57-361.21)	**0.046**	71.38 (1.19-431.68)	0.691	0.85 (0.02-23.35)	**0.010**
≥IB2	36	36.80 (1.91-253.59)		94.01 (1.09-306.77)		0.37 (0.04-42.91)	
Pathological type							
Squamous carcinoma	61	48.86 (1.57-361.21)	0.920	101.99 (1.09-431.68)	0.085	0.53 (0.02-50.00)	0.444
Adenocarcinoma	16	39.63 (3.68-197.66)		51.92 (4.37-242.26)		0.69 (0.06-33.33)	
Differentiation							

2 (N=77).

Well and moderately differentiated	50	51.90 (3.68-361.21)	0.364	64.31(1.09-431.68)	**0.002**	0.88 (0.06-31.71)	**0.000**
Poorly differentiated	27	42.47(1.57-260.11)		152.49 (5.91-368.08)		0.27 (0.02-42.91)	
Pelvic lymph node metastasis							
Negative	40	81.68 (9.52-253.59)	0.421	65.70 (1.09-431.68)	0.476	0.78 (0.14-42.91)	0.281
Positive	21	45.14 (3.68-361.21)		83.67 (1.68-395.47)		0.77 (0.02-23.35)	
Tumor size							
≤ 2cm	52	59.92 (1.57-361.21)	**0.034**	101.99 (1.19-431.68)	0.306	0.58 (0.02-42.91)	0.286
> 2cm	25	34.41 (1.91-203.59)		69.23 (1.09-291.58)		0.35 (0.04-31.71)	
Lymphovascular space invasion							
Negative	48	56.12 (1.57-361.21)	**0.039**	80.66 (1.19-431.68)	0.328	0.71 (0.02-50.00)	**0.013**
Positive	29	38.12 (1.91-121.64)		114.26 (1.09-299.27)		0.41 (0.04-20.00)	