Peer Review File

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Review Comments:

The authors have investigated the expression of CLDN18.2 in advanced gastric cancer (GC) of signet-ring cell carcinoma (SRCC). They found CLDN18.2 frequently and highly expressed in SRCC of advanced GC. They further performed NGS analysis and found significant correlation between CLDN 18.2 and GRIN2A. Although the study is well-conducted and I consider this paper has potential of publication, I have following comments. The most incomprehensible point for me is why the authors only subjected SRCC? Although WHO classification indicated SRCC as 50% of signet-ring cells, its pathological diagnosis often varies by pathologist. Actually, I cannot agree the diagnosis of SRCC in provided digital photographs in Figure 1. I recommend replacing the word "SRCC" to "diffuse type" of advanced GC. If the authors persist "SRCC", the pathological diagnosis of SRCC in subjected series should be confirmed by at least two expert pathologists and should describe these matters in Materials and Methods. In addition, expression of CLDN18.2 should be assessed separately in typical signet-ring cells and other cancer cells and should demonstrate typical figures of signet-ring cells. Although only the Stages (III or IV) were provided in Table 1, detailed TMN factors and detailed therapy information involving adjuvant and neoadjuvant therapy should be presented. Although the authors used FFPE of GC tissues, all samples are surgically resected specimens? Status of surgery (curative resection or not) should be also documented. The findings regarding GRIN2A are interesting. However, supporting evidence seems weak because small number of samples. This should be documented as limitation.

Comment 1: *The most incomprehensible point for me is why the authors only subjected SRCC? Although WHO classification indicated SRCC as 50% of signet-ring cells, its pathological diagnosis often varies by pathologist. Actually, I cannot agree the diagnosis of SRCC in provided digital photographs in Figure 1. I recommend replacing the word "SRCC" to "diffuse type" of advanced GC. If the authors persist "SRCC", the pathological diagnosis of SRCC in subjected series should be confirmed by at least two expert pathologists and should describe these matters in Materials and Methods. In addition, expression of CLDN18.2 should be assessed separately in typical signet-ring cells and other cancer cells and should demonstrate typical figures of signet-ring cells.*

Reply 1: Thank you for your excellent comments and suggestions. We agree that pathological diagnosis of SRCC varies by pathologist. Therefore, we asked two independent pathologists to re-diagnose the samples. The diagnosis of SRCC were confirmed by them all and typical photographs have been provided in Figure 1A in the text. The detailed information of the percentage of signet-ring cells and the expression of CLDN18.2 in typical signet-ring cells and other cancer cells was shown in Table 1 below. The results showed that CLDN18.2 expression in signet ring cells and other tumor cells was both high in SRCC patients, and that might be the reason for the

increased invasive potential of SRCC. Thanks to the reviewer for the brilliant comments on IHC photographs. Our samples have been confirmed to be SRCC and the photographs of CLDN18.2 staining intensity were replaced with typical SRCC photographs.

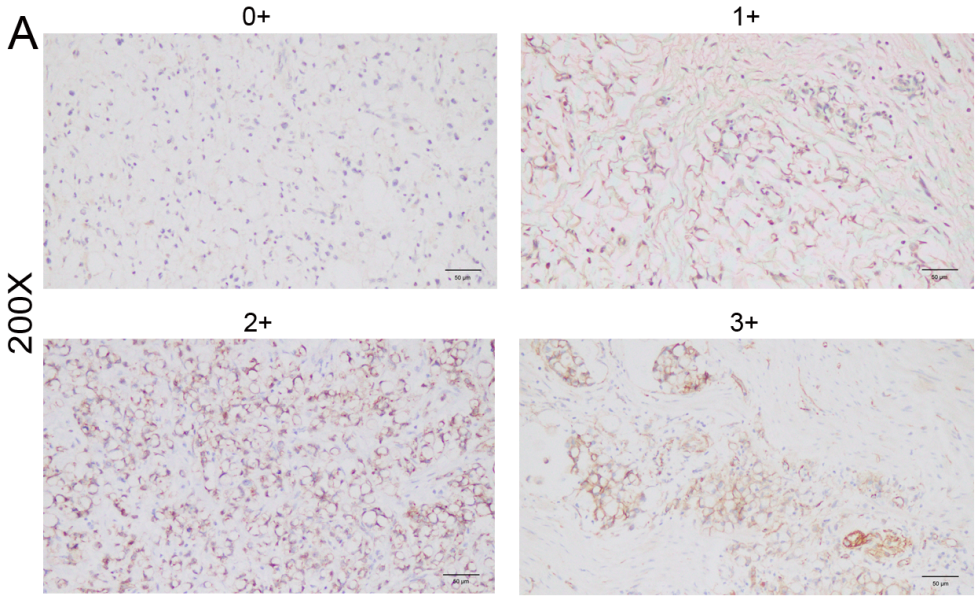

Figure 1A. Micrographs of representative stained tissues: 0+, 1+, 2+ and 3+ staining intensity. The magnification was 200X.

Table 1. CLDN18.2 expression in signet ring cells and other tumor cells in SRCC.

Percentage of signet-ring cells	Total cases	CLDN18.2 staining intensity	Cases	
			Signet-ring cells	Other cancer cells
51-70%	35	0	4	4
		1+	10	10
		2+	10	11
		3+	11	10
71-90%	70	0	1	1
		1+	12	12
		2+	27	28
		3+	30	29

Changes in the text: We have modified our text as advised (see Page 8, line 9 and Figure 1A).

Comment 2: *Although only the Stages (III or IV) were provided in Table 1, detailed TMN factors and detailed therapy information involving adjuvant and neoadjuvant therapy should be presented.*

Reply 2: Thank you for your helpful comments. We had 92 stage III patients and 13 stage IV patients. Of all the stage III patients, 62 were T3, 30 were T4, 6 were N1, 18 were N2, 68 were N3. Detailed TMN information and the relation with CLDN18.2 expression were shown in Table 2 below (added as Supplementary Table 1). There

is no correlation between CLDN18.2 expression and TNM stage. Patients at stage III were all administrated first-line 5-FU-based adjuvant chemotherapy after D2 gastrectomy, while patients at stage IV were treated by first-line 5-FU-based palliative chemotherapy. None of them had radiotherapy, chemotherapy or other medical intervention before specimen collection. Detailed explanation had been added in the text.

Table 2. The relation between CLDN18.2 expression and TNM stage in stage III patients.

Stage	TNM		Total cases (%)	CLDN18.2 expression			
				Staining intensity ≥2+ in ≥40% of cells (%)	P value	Staining intensity ≥2+ in ≥90% of cells (%)	P value
III	T	T3	62 (67.4)	40 (64.5)	0.674	15 (24.2)	0.108
		T4	30 (32.6)	18 (60.0)		3 (10.0)	
	N	N1	6 (6.5)	2 (33.3)	0.293	0 (0.0)	0.326
		N2	18 (19.6)	12 (66.7)		5 (27.8)	
		N3	68 (73.9)	44 (64.7)		13 (19.1)	

Changes in the text: We have modified our text as advised (see Page 6, line 22 and Page 9, line 21).

Comment 3: *Although the authors used FFPE of GC tissues, all samples are surgically resected specimens? Status of surgery (curative resection or not) should be also documented.*

Reply 3: Thank you. In our study, samples from stage III patients were curative surgical specimens, while samples from stage IV patients were palliative surgical specimens or gastroscope specimens. We have supplemented the status of surgery in the manuscript.

Changes in the text: We have modified our text as advised (see Page 7, line 1).

Comment 4: *The findings regarding GRIN2A are interesting. However, supporting evidence seems weak because small number of samples. This should be documented as limitation.*

Reply 4: Thank you for your constructive comments. The relatively small sample size is the limitation for the study of GRIN2A. This has been addressed accordingly in the revised manuscript.

Changes in the text: We have modified our text as advised (see Page 14, line 14).