Lymphoid Enhancer-Binding Factor 1 (LEF1) immunostaining as a surrogate of β-Catenin (*CTNNB1)* mutations

Running title: LEF1 immunostaining and *CTNNB1* mutations

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Abstract:

Activating mutations affecting exon 3 of the β-Catenin (*CTNNB1*) gene result in constitutive activation of WNT signalling and are a diagnostic hallmark of several tumour entities including desmoid-type fibromatosis or define clinically relevant subtypes such as in endometrioid carcinoma. In a diagnostic setting, β-Catenin immunohistochemistry is widely used as a surrogate of *CTNNB1* mutations, but is often difficult to assess in practice, given that the characteristic nuclear translocation may be focal or hard to distinguish from spillover of the normal membranous staining.

We therefore assessed Lymphoid Enhancer-Binding Factor 1 (LEF1) immunohistochemistry, a nuclear marker of WNT activation as a potential surrogate of *CTNNB1* mutations. Across a variety of entities characterised by *CTNNB1* mutations as a putative driver we found diffuse and strong expression of LEF1 in 77% of cases. In a cohort of endometrial carcinomas (n=255) LEF1 was accurate to predict *CTNNB1* mutations in 85% ($p<0.001$), while β-Catenin was accurate in 76% ($p<0.001$). Irrespective of tumour type, we found LEF1 immunostaining to be easier to interpret than β-Catenin immunostaining in 54% of cases, more difficult in 1% and equally easy to interpret in the remainder.

We conclude that LEF1 immunostaining is a highly useful surrogate marker of *CTNNB1* mutations in lesions which are driven by WNT signalling, favourably complementing β-Catenin immunohistochemistry and outperforming the latter as a single marker.

Introduction

Mutations in the exon 3 of the β-Catenin (*CTNNB1*) result in constitutive activation of WNT signalling and thereby contribute to tumorigenesis. They are recognized as a hallmark of several types (including desmoid-type fibromatosis, pilomatricomas, adamantinomatous craniopharyingiomas). Herein, b-Catenin directed immunohistochemistry serves as a diagnostic marker to distinguish them from morphological mimickers. Activating *CTNNB1* mutations result in nuclear translocation of β-Catenin protein, which under physiological conditions locates to adherens junctions. This nuclear translocation can be visualised immunohistochemically, in that *CTNNB1* mutant tumours display a combined membranous and nuclear β-Catenin staining, in contrast to the isolated membranous staining found in non-mutated tumours or non-neoplastic tissue.

In the diagnostic routine, β-Catenin immunostaining is widely used as surrogate marker for *CTNNB1* mutations across a variety of lesions, because of lower costs and faster turnaround times as compared to gene sequencing. One such entity is for example solid-pseudopapillary neoplasm of the pancreas where a positive mutation can be very helpful in the diagnostic setting, even in samples obtained by endoscopic ultrasound-guided fine needle aspiration [1]. Another one is desmoid-type fibromatosis where it was shown that the identification of *CTNNB1* mutations can be exploited diagnostically in difficult lesions [2].

Sporadic endometrial carcinomas harbour *CTNNB1* mutations in about 20% [3]. Recently, it was reported that these mutations identify patients with an increased risk of recurrence and in a follow-up paper, the same group reported that β-Catenin immunostaining may be used as a screening tool to identify the tumours which should undergo *CTNNB1* sequencing [3] [4]. This was confirmed in a large meta-

analysis which found that indeed β-Catenin immunostaining can be used as a screening tool for *CTNNB1* mutations [5].

Currently, there are more than 60000 new cases with endometrial cancer diagnosed each year in the United States, according to the SEER database which corresponds to 7% of all newly diagnosed cancer cases in women[6]. The incidence and rate of death is rising, thus identifying patients at risk for recurrence and tumour progression is urgently needed.

Because of the frequently focal nature of nuclear translocation and nuclear spillover of membranous staining, β-Catenin immunohistochemistry is notoriously difficult in the readout and requires significant expertise [7]. Therefore, a new marker which is easier to interpret would be very helpful. This prompted us to assess the utility of LEF1 (lymphoid enhancer binding factor 1), a downstream mediator of WNT signalling currently used as a diagnostic marker for chronic lymphoid leukaemia [8], as a potential immunohistochemical surrogate of *CTNNB1* mutations.

Material and methods

Tumour selection

Eight patients with a fibromatosis of desmoid type and three with a peripheral superficial fibromatosis were included in the study.

Additionally, we included tumours which underwent sequencing for diagnostic or predictive purposes and in which a *CTNNB1* mutation was found. In total, there were 19 such cases including different colon and lung carcinomas and one solid pseudopapillary tumour of the pancreas.

There were 255 endometrial carcinomas included of which 85 underwent *CTNNB1* mutation analysis and 64 underwent NGS sequencing using the Illumina TruSight Oncology 500 panel. The study was performed in accordance with the Swiss Federal Act on Research involving Human Beings and with approval of the Ethics Commission of the Canton of Bern (KEK 2014-200 and 2017-1189).

Tissue microarray construction

A tissue microarray (TMA) was constructed as previously described [9]. In total, 255 cases of endometrium carcinoma were included. All clinical and histopathological data of the cohort were previously outlined [10]. Three representative punches (1mm core) were taken per carcinoma and two additional ones for DNA extraction.

Immunohistochemistry

All cases with endometrium carcinoma of which mutational status was available (n=139) and all other cases (n=26) were immunohistochemically stained with β-Catenin and Lymphoid enhancer-binding factor 1 (LEF1; n=165), using the Leica Microsystems Bond Max Stainer. Formalin-fixed, paraffin-embedded 4 µm thick

slides were stained with β-Catenin (CellMarque, clone 14, order nr 224M-15, dilution 1:400) and LEF1 (Abcam, clone EPR2029Y, order nr ab137872, dilution 1:100). Staining was assessed independently by two expert pathologists, blinded to molecular data (EH and MSD). Any nuclear positivity of β-Catenin was considered positive. LEF1 was considered positive when more than 50% of tumour cells were strongly nuclear positive. In incongruent cases, a consensus was reached in a second session. Both pathologists also assessed the difficulty to decide whether a staining was positive or negative.

DNA extraction

All cases underwent DNA extraction as described elsewhere to undergo molecular analysis [11]. Briefly, tumour tissue was identified by a molecular pathologist (MSD) and the area of interest was marked on a haematoxylin and eosin (H&E) stained slide. The area of interest was identified on the formalin fixed and paraffin embedded tissue (FFPE) block and punched.

Sanger Sequencing

75 cases were analysed by sanger sequencing. A fragment encompassing exon 3 of the CTNNB1 gene was amplified by PCR using the primer pair 5'-GCC ATG GAA CCA GAC AG-3' and 5'-TTC CCA CTC ATA CAG GAC TT-3' and analysed by Sanger Sequencing using a Genetic Analyzer (GA3500, Thermofisher).

Next generation sequencing

18 cases underwent comprehensive NGS sequencing with various panels over a 6 years time span. 64 endometrial carcinomas underwent sequencing with the

TruSight oncology 500 panel of Illumina (TSO500), encompassing 523 genes and allowing us not only to look at *CTNNB1* mutations but at various other genes in the WNT signalling pathway (*APC, AXIN1, AXIN2, CSNK1A1, EP300* and *PPP2R1A*).

Results

β-Catenin and LEF1 in diagnostic setting of fibromatoses and solid pseudopapillary tumour of the pancreas

Fibromatoses of the desmoid type carried *CTNNB1* mutations in 63% of cases (5/8). β-Catenin and LEF1 were similarly positively expressed in all mutated and two wildtype cases. Superficial fibromatoses were all *CTNNB1* wildtype and immunohistochemically, β-Catenin and LEF1 were again similarly expressed (positive in one, negative in two cases). The LEF1 readout was easier in all cases (fig. 01 a-c).

The solid pseudopapillary tumour of the pancreas was *CTNNB1* mutated and expressed both immunohistochemical markers. Again, the readout of LEF1 was considered easier (fig. 01 d-f).

β-Catenin and LEF1 in endometrial carcinomas

CTNNB1 mutations were found in 25 out of 139 cases. As expected, immunohistochemistry correlated excellently with mutational status for β-Catenin ($p<0.001$) as well as for LEF1 ($p<0.001$). The overall sensitivity and specificity of β-Catenin was ($p<0.720$ and $p<0.229$) and of LEF1 was ($p<0.64$ and $p<0.095$).

The readout of LEF1 however was easier in 54% cases (n=70), comparable to β-Catenin in 45% of cases (n=58) and more difficult in 1% of cases (n=2) (fig. 02).

β-Catenin and LEF1 immunohistochemistry were both able to predict relapse free survival (RFS) ($p<0.05$) and in addition, LEF1 also predicted overall survival (OS)

($p<0.05$) in patients with endometrial carcinoma whereas this was not possible with β-Catenin staining (fig. 03).

CTNNB1 mutations were also significantly correlated with OS (fig. 04), a finding which could be confirmed by the TCGA Dataset from cBioPortal [12,13] including 542 endometrial carcinomas (fig. 05). RFS was not predicted by CTNNB1, probably due to an outlier (fig. 04).

When running a multivariate analysis for OS and RFS including MSI, POLE-Status, tumour grade and tumour stage, this effect was lost for β-Catenin as well as for LEF1 - only tumour grade and stage remained significant.

β-Catenin and LEF1 in various neoplasms

CTNNB1 mutated neoplasms were a heterogenous group of various entities (Adenocarcinoma of the lung n=5, colon carcinoma n=5, malignant melanoma n=3, adrenocortical carcinoma, prostate carcinoma, pancreatic adenocarcinoma, medulloblastoma, neuroendocrine carcinoma of the lung (each 1x; total n=18). Immunohistochemistry for β-Catenin predicted mutational status correctly in 89% of cases, whereas LEF1 only in 50%. When there was a concurrent other strong driver mutation such as *KRAS, EGFR* or *TP53* present, LEF1 was negative in 100% while β-Catenin was negative in 25% of patients (fig. 01 g-i).

Discussion

The study explored the utility of LEF1 immunohistochemistry as a surrogate marker for *CTNNB1* mutations in various human neoplasms in different diagnostic and predictive settings. The *LEF1* gene belongs to the TCF/LEF (T cell factor/lymphoid enhancer factor) gene family. LEF1 physically interacts with β-Catenin when the latter is translocated to the nucleus and mediates WNT signalling. LEF1 overexpression has been described in several tumour types harbouring *CTNNB1* mutations, such as cribriform-morular variant of papillary thyroid carcinoma [14] or deep penetrating melanocytic naevi [15], but has not been assessed systematically as surrogate for *CTNNB1* mutations across tumour types.

β-Catenin and LEF1 as a diagnostic marker

CTNNB1 mutations can be found in the majority of sporadic fibromatoses [16]. They are known to carry this mutation in about 90% of cases which is used diagnostically with β-Catenin immunohistochemistry as a surrogate marker. Normally, β-Catenin staining patterns are membranous and cytoplasmic. In case of a CTNNB1 mutation or canonical WNT pathway activation, β-Catenin gets translocated to the nucleus where it activates downstream transcriptional programs [17]. As previously reported, we could identify a nuclear positivity of β-Catenin in most deep fibromatoses. What is new, is that we also stained for LEF1 which is downstream of β-Catenin and here, we saw that this marker was a lot easier to evaluate in all cases. Interestingly, this holds also true for superficial fibromatoses which do not carry a *CTNNB1* mutation but nevertheless have a nuclear positivity of β-catenin [18,19].

Prognostic role of β-Catenin and LEF1 in endometrial carcinomas

The role of β-Catenin immunohistochemistry has been studied in the past. It was demonstrated that about 40% of endometrial carcinomas show a nuclear β-Catenin expression in less than 10% of tumour cells [4]. Although the authors concluded that β-Catenin can reliably predict mutational status of *CTNNB1*, a marker which is somewhat easier to evaluate and does not require a lot of expertise to achieve a reliable readout would be obviously very helpful. Therefore, we explored the role of LEF1 and found that the readout is easier than that of β-Catenin to predict a *CTNNB1* mutation. Two expert pathologists (EH and MSD) evaluated whether LEF1 was easier to evaluate as β-Catenin and both came independently to the same conclusion which is obviously (as many decisions in pathology) to a certain degree subjective. In fact, the readout was easier in 50% of cases and equal to β-Catenin in another 29%. Nevertheless, as illustrated in fig. 02, we think it is fair to state that the readout of LEF1 is in general much easier than that of β-Catenin.

LEF1 was upregulated in the tumour front and in areas on epithelial-mesenchymal transition which fits well in the concept of an activated WNT signalling pathway under these circumstances [20]. Therefore, a 50% cut-off is helpful in distinguishing the cases where only WNT is active whereas the other ones where a *CTNNB1* mutation is actually present in the neoplasm.

Ruz-Caracuel et al. compared the prediction of *CTNNB1* exon 3 mutations by immunohistochemistry for β-Catenin and LEF-1 in low-grade, early-stage endometrial endometrioid carcinoma and found β-Catenin to be both more specific for the presence of mutations [21]. An adverse effect of the presence of a *CTNNB1* mutation in these tumours has been reported as well [4,21]. A subgroup analysis of

early endometrioid carcinomas of our cohort revealed a nonsignificant trend towards an adverse outcome in the presence of a *CTNNB1* mutation, confirming these results. However, when we included all carcinomas into the analysis, the presence of a *CTNNB1* mutation was a favourable sign in our data which we saw also in the TCGA dataset [22].

Nevertheless, the difficulty of the β-Catenin immunohistochemistry readout persists and while one group does not comment on that topic, the other study gives an indirect hint since they reported only a poor correlation between the β-Catenin and *CTNNB1* mutation [4], underscoring the need of a better surrogate marker.

The reliable identification of *CTNNB1* mutations by immunohistochemistry is also underscored by the recently published PORTEC-4a trial which stratified mismatch repair deficient endometrial carcinomas based on their mutational status into different risk groups [3].

Inconclusive β-Catenin and LEF1 immunohistochemistry

A variety of different malignant neoplasms (carcinomas, glial and melanocytic origin) and a known deleterious *CTNNB1* mutation were also stained for β-Catenin and LEF1. Interestingly, if a concurrent strong other driver was present, neither β-Catenin nor LEF1 showed a nuclear expression, defying the concept of nuclear positivity in the presence of a mutation. This has been previously observed in 6 endometrium carcinoma cases with *KRAS*, *RET* and *TP53* mutations[4]. The reason for this is still unknown, however, it might be possible that in the presence of another strong tumour driver, the WNT pathway is not active even though there is a *CTNNB1* mutation. This certainly warrants further investigations in this direction and might

also play a role in tumour resistance, epithelial-mesenchymal transition and progression.

Conclusion

We report immunohistochemical and prognostic results of LEF1 in a series of 139 endometrial carcinomas and compare them to the staining results of β-Catenin. The CTNNB1 mutational status always serves as the ground truth in all cases. In addition, we explore the role of LEF1 in other diagnostic challenging lesions which are known to harbour CTNNB1 mutations, such as fibromatoses and a solid pseudopapillary tumour of the pancreas. Lastly, we explore the role of LEF1 and β-Catenin in a series of various malignant neoplasms, most of which also demonstrate a concurrent other driver mutation.

We demonstrate that LEF1 immunohistochemistry can be used in the diagnostic and predictive scenario and predicts CTNNB1 mutations as good as β-Catenin. And while the β-Catenin readout requires a lot of expertise, the readout of LEF1 is much easier. The negativity of immunohistochemistry of β-Catenin and LEF1 in the presence of a CTNNB1 mutation and a strong known other driver mutation might be a hint that the EMT pathway is not active which might play a role in tumour progression and patient management.

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Author contributions:

MSD and EH conceived the study and wrote the manuscript. All authors analysed data and approved the final version of the manuscript.

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Figure 01:

A-C: Desmoid type fibromatosis: A: HE showing classic bland spindle cell morphology; B: nuclear positivity of LEF1, C: nuclear and cytoplasmic positivity of β-Catenin. D-F: Solid pseudopapillary tumor of the pancreas (SPN): dD: HE staining, SPN on the right side, normal pancreas on the left; E: nuclear positivity of LEF1 in SPN while normal pancreas is negative, F: nuclear and cytoplasmic positivity of β-Catenin in SPN as compared to membranous positivity in normal pancreatic tissue. G-I: Intestinal adenocarcinoma: G: intestinal differentiated adenocarcinoma; H: infiltrative carcinoma is negative for LEF1; I: strong nuclear and cytoplasmic positivity of the adenocarcinoma for β-Catenin

Figure 02:

LEF1 and β-Catenin in endometrial carcinoma. A: negativity of LEF1; B: membranous positivity of β-Catenin and partial nuclear positivity, rendering a classification difficult; C: strong nuclear positivity of LEF1; D: strong nuclear, cytoplasmic and membranous positivity of β-Catenin

Figure 03:

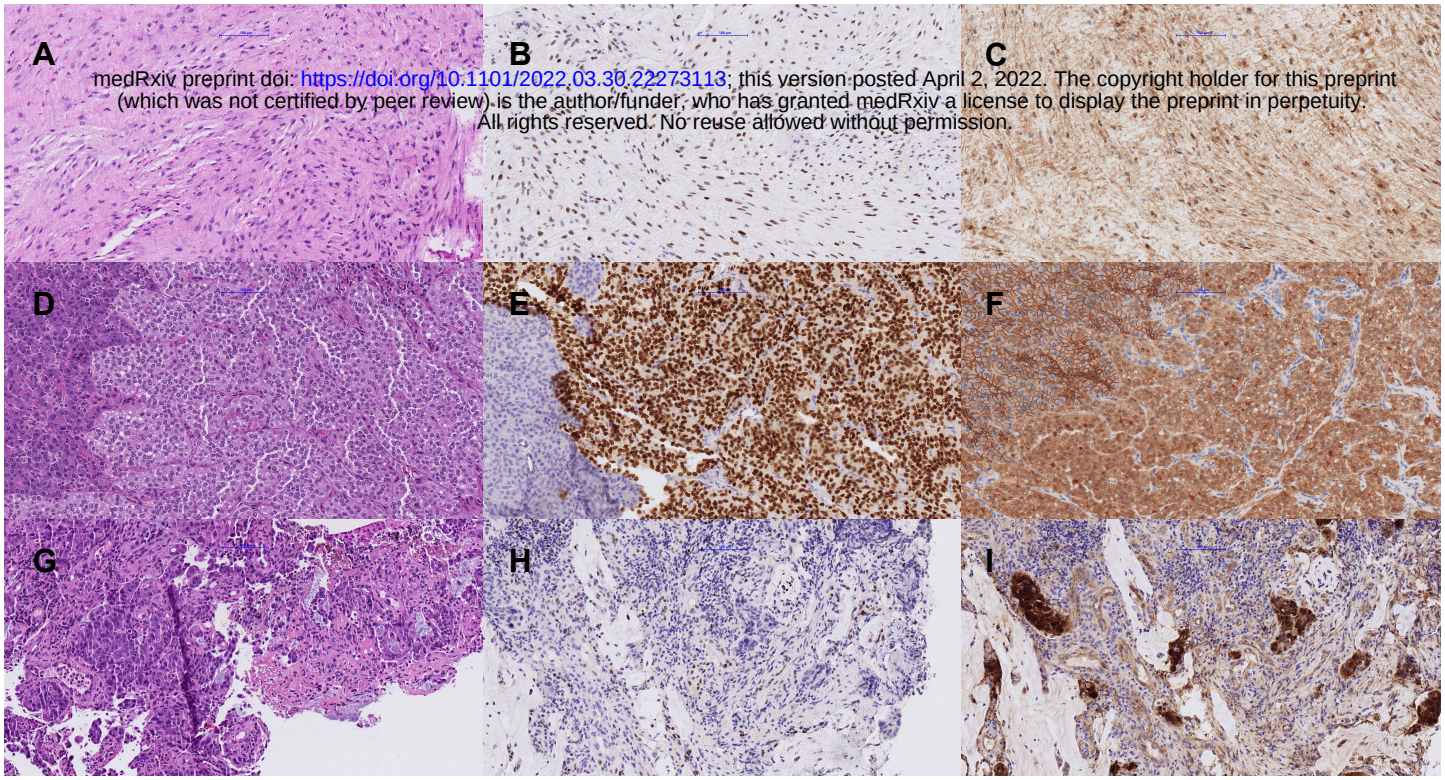
Survival curves LEF1 and β-Catenin immunohistochemistry; A: LEF1 relapse free survival (RFS)($p<0.05$); B: β-Catenin RFS ($p<0.05$); C: LEF1 overall survival (OS)($p<0.05$); D: β-Catenin OS ($p<0.13$)

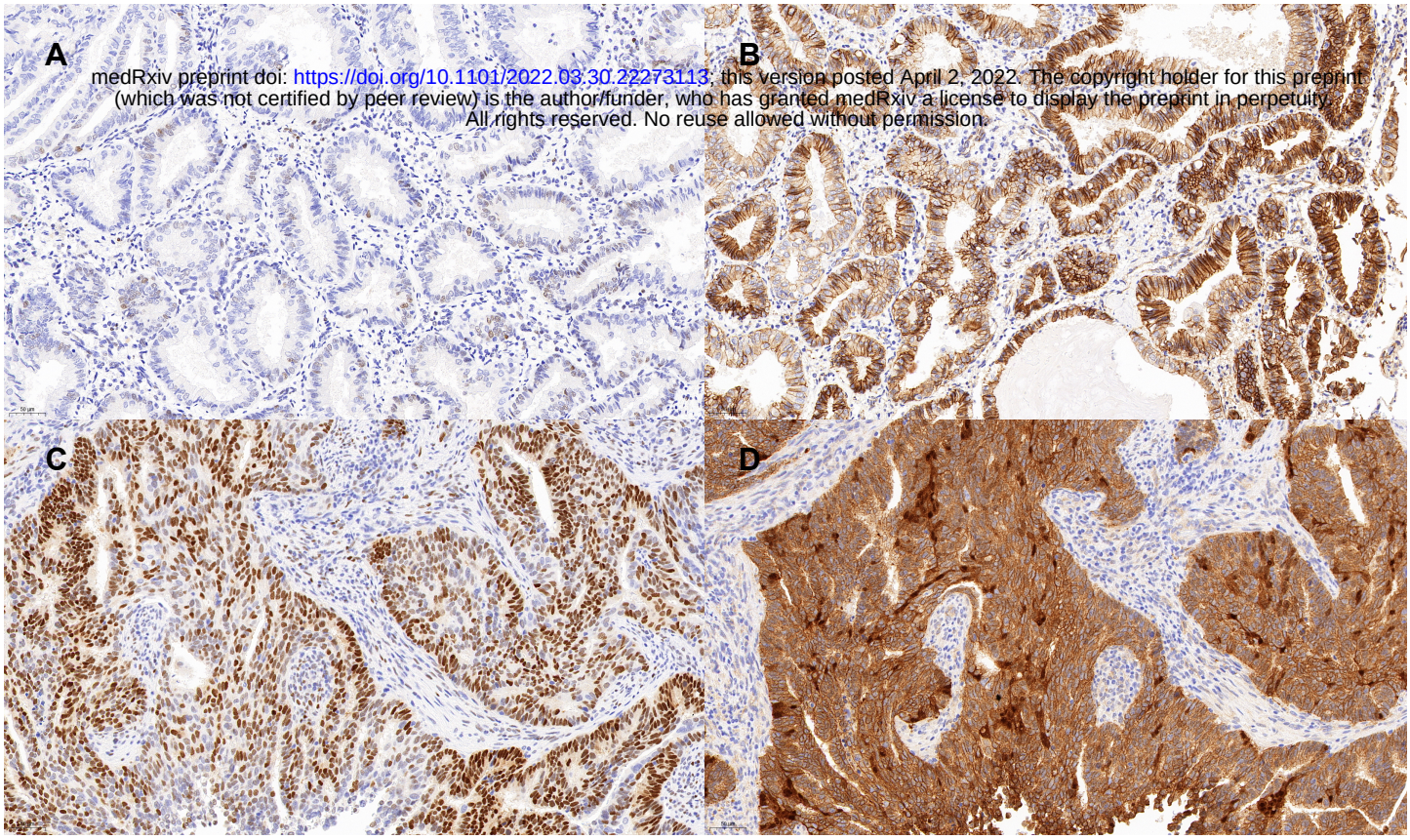
Figure 04:

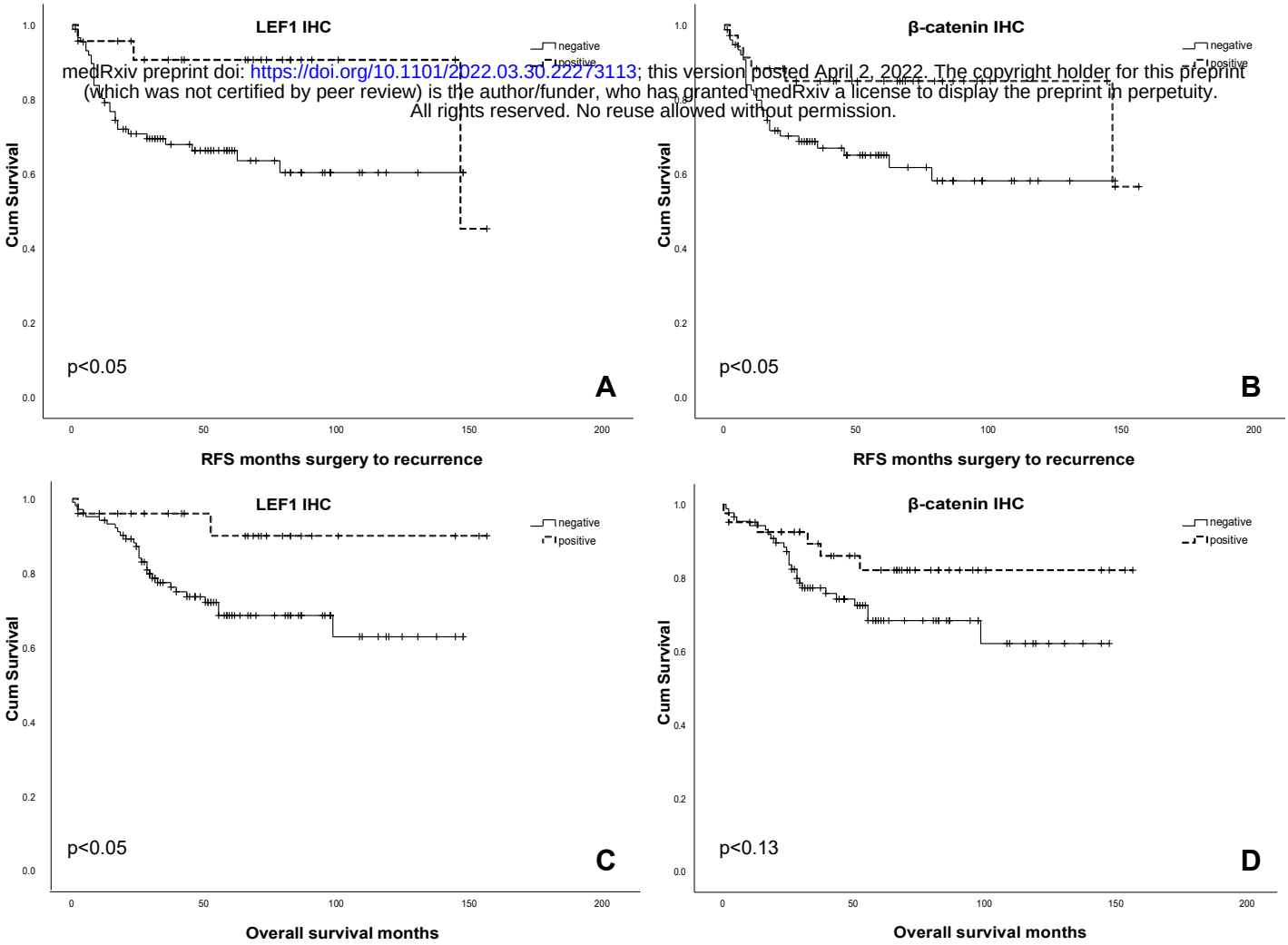
Survival curves *CTNNB1*; A: relapse free survival ($p<0.27$); B: overall survival ($p<0.05$)

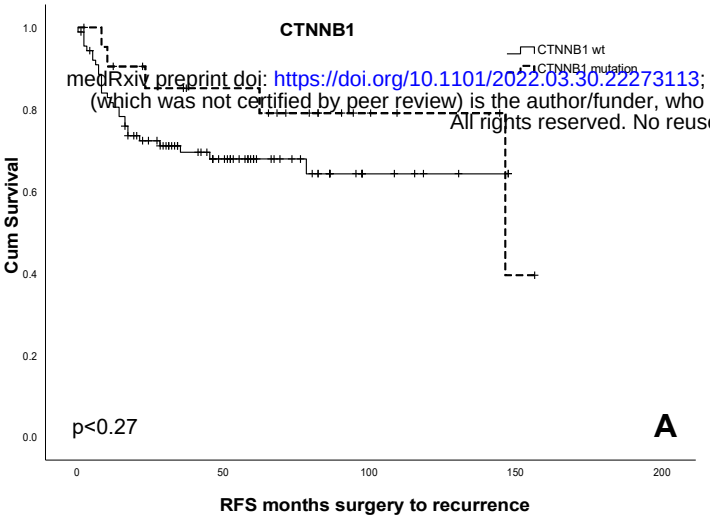
Figure 05:

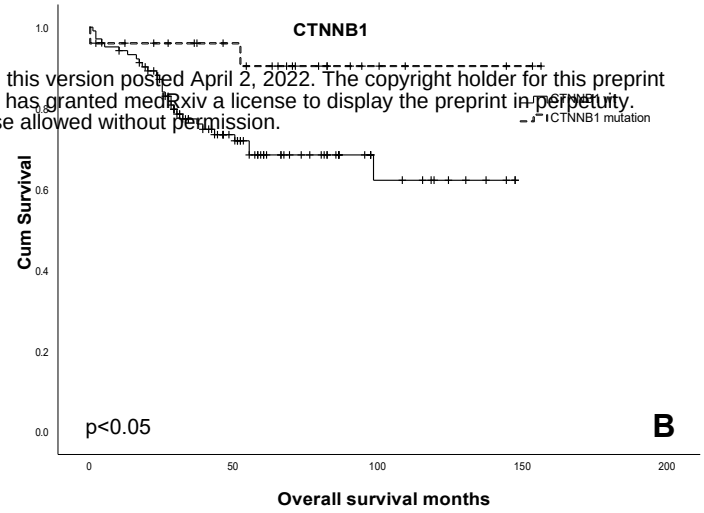
Overall survival by *CTNNB1* mutation status in the TCGA dataset ($p<0.05$).

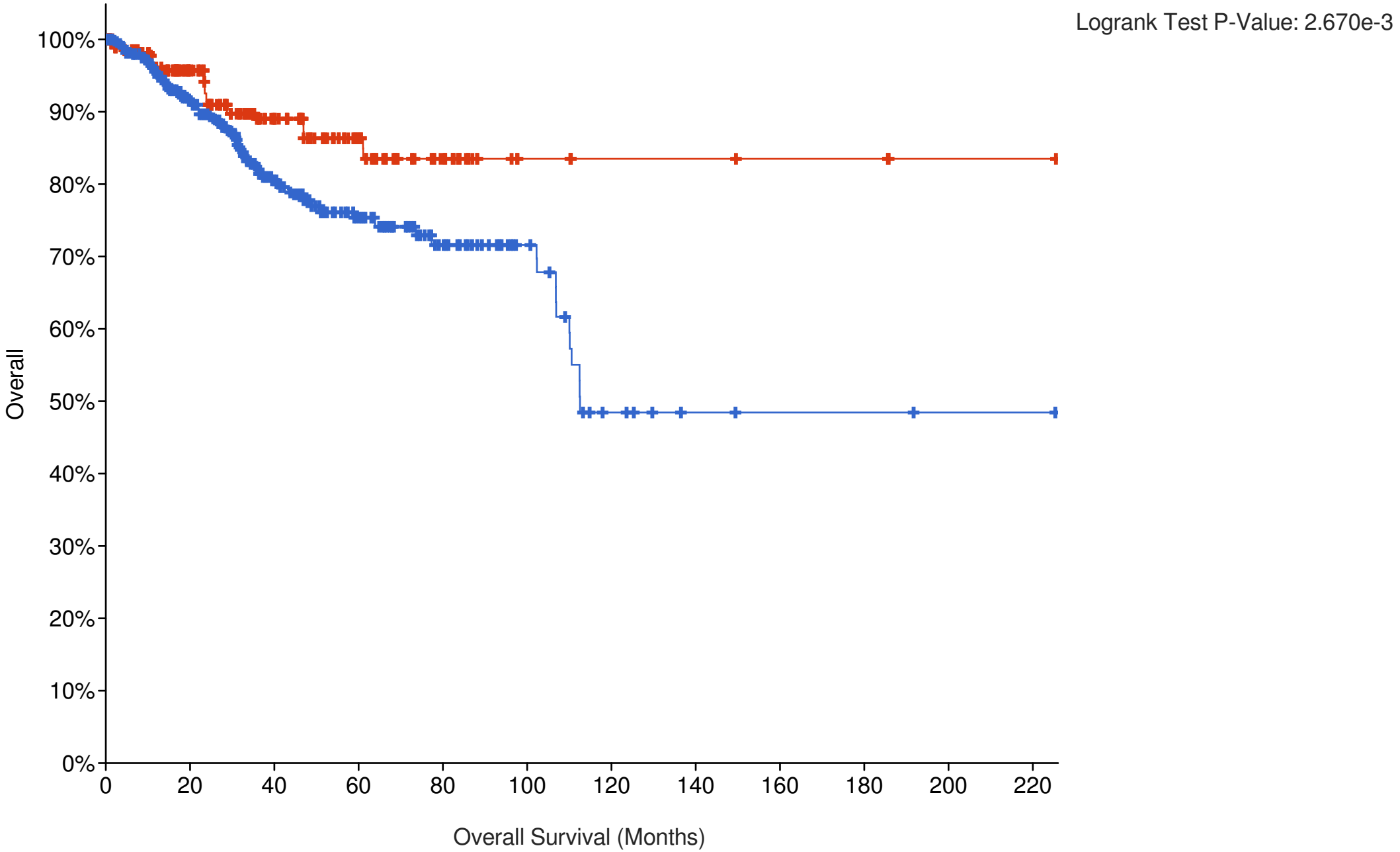








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Overall Survival (Months)
Overall
Altered group
Unaltered group