

Prognostic value of cyclin A2 and B1 expression in lung carcinoids



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Summary

Carcinoid classification in the lung is still based on morphological criteria. Although there are many studies investigating the role of Ki-67 proliferation index in the classification of lung neuroendocrine tumours, it is still not used in routine diagnostics. Interestingly, cyclins, which have a crucial role in controlling the cell cycle, have not been thoroughly studied in lung neuroendocrine tumours. The aim of our study was to investigate the correlation of cyclin A2 and B1 expression with prognosis, Ki-67 proliferation index, and carcinoid morphology. A cohort of 134 resected typical and atypical carcinoids was stained with antibodies against Ki-67, cyclin A2 and B1. The positive nuclear reaction was assessed in hot spot areas and expressed as the percentage of tumour cells. Univariate analyses found the highest relative hazard between low and high cyclin A2 expression both with respect to overall survival [hazard ratio (HR)=16; 95% confidence interval (CI) 4.8–51; $p=0.000054$], and relapse (HR=8; 95% CI 3.1–21; $p=0.00002$). In multivariate analysis for overall survival cyclin A2 (HR=10; 95% CI 2.5–100; $p=0.0082$) and B1 (HR=6.5; 95% CI 1.5–35; $p=0.02$) remained significant when adjusted for other risk factors, whereas Ki-67 was no longer significant (HR=0.64; 95% CI 0.003–5.5; $p=0.65$). This suggests that Ki-67 is closer to conventional risk factors for survival than cyclin A2 and B1. Furthermore, the analysis revealed 4 mitoses per 2 mm² as a more powerful prognostic cut-off than currently accepted 2 mitoses. We have clearly demonstrated that application of cyclin A2 and cyclin B1 might bring additional value regarding the overall and progression-free survival of patients with carcinoids of the lung.

Key words: Carcinoid; lung; cyclin; Ki-67; mitosis.

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INTRODUCTION

Cell cycle progression is regulated by the interaction of cyclin, cyclin dependent kinases (CDKs) and CDK inhibitors.¹ There are more than 20 different CDKs and cyclins, but only some cyclin-CDK complexes are important in progression and control of the cell cycle.² Therefore, it is not surprising that their molecular changes and changes in their expression have been detected in many tumours.^{3,4} However, different cyclins are differently expressed and regulated in different cell cycle phases.

After binding of cyclin A2 to CDK2, DNA replication is induced, and the cell passes through S phase. Furthermore, the cyclin A2-CDK2 complex is essential for the progression of G2 phase into mitosis.⁵ Cyclin A is localised in the nucleus during S phase, controlling DNA synthesis.⁶ In G2 phase it moves to centrosomes where it regulates cyclin B-CDK1 complex activation.⁷ Cyclin B binds to CDK1 at the end of S phase and the complex initiates mitosis (M phase); thereafter the complex is degraded.⁸ Since the role of the cyclin B-CDK1 complex in mitosis is crucial, it is not unexpected to see its dysregulation in many tumours. Several studies demonstrated high cyclin B1 expression in different carcinomas, which was associated with aggressiveness of tumour, and might serve as a prognostic marker.^{9–11} It can be present in the cytoplasm and in the nucleus, the latter associated with poorer prognosis (as demonstrated in breast and oesophageal carcinomas).^{12,13} On the other hand, Zheng *et al.* showed that high expression of cyclin B1 is present in ovarian tumours with low malignant potential, but not in ovarian carcinomas.¹⁴ Cyclin D1 accumulates in the nucleus in G1 phase, and after dimerisation with CDK4 and CDK6 regulates transition from G1 to S phase. Independently of CDK it also regulates cell differentiation, growth and proliferation. Increased expression of cyclin D1 is present in different carcinomas for which it is also a poor prognostic marker.^{15–17} Igarashi *et al.* demonstrated a different expression of cyclin B1 in typical (TC) versus atypical carcinoids (AC), while no

significant difference in cyclin D1 was observed.¹¹ However, they did not look closely into this, and the role of cyclins A2 and B1 in pulmonary carcinoids until now has not been evaluated.

In the 2015 World Health Organization (WHO) classification, TC and AC, together with large cell neuroendocrine carcinoma and small cell carcinoma, are grouped into neuroendocrine tumours (NET) of the lung.¹⁸ TC represents the low-grade end of this spectrum and AC is a tumour of intermediate malignancy. Both represent 1–2% of all primary lung malignancies; however, they are the most common primary lung malignancies in children and late adolescents.¹⁹ Distinction between TC and AC is still based on morphology alone: tumours with neuroendocrine morphology (i.e., rosettes, trabeculae, nests), <2 mitoses per 2 mm², and absence of tumour necrosis are classified as TC, while tumours with 2–10 mitoses per 2 mm², and/or presence of tumour necrosis, should be classified as AC.¹⁸ Differentiation between TC and AC is important not only because AC more often have distant metastases and worse prognosis, but also because therapy protocols might differ. Ki-67 as a proliferation marker was introduced in the last WHO classification as a tool to distinguish carcinoids (with proliferation index <20%) from high-grade NET [small cell lung cancer (SCLC) and large cell neuroendocrine carcinoma (LCNEC)] where proliferation index is >40%.¹⁸ This is in line with the European Neuroendocrine Tumour Society expert consensus and recommendations, where Ki-67 was found to be useful in small biopsies as a tool for separating carcinoids from high-grade NET.¹⁹ Different studies analysed Ki-67 expression in carcinoids, applying a range of cut-off values (2.5–7.0%); but most of them were not able to clearly separate TC and AC.^{20,21}

As previously demonstrated, there is some inter-observer variability in carcinoid classification using only morphological criteria.²² Furthermore, in small biopsies it is difficult to assess all histological criteria, since the area of the whole tumour sample is often smaller than 2 mm². Even with the help of Ki-67, proper prognostic stratification is not always possible. This is further hampered by the existence of tumour heterogeneity, as with many other prognostic markers.

The aim of this study was to assess the prognostic value of cyclins A2 and B1 in a large cohort of pulmonary carcinoids, to compare them with morphological criteria and Ki-67, and to correlate this with survival data.

MATERIAL AND METHODS

Patients and tumour samples

Cases with a diagnosis of pulmonary carcinoid were selected from the archives of the Institute of Pathology, Medical University of Graz, and the Institute of Pathology, School of Medicine University of Zagreb, in the time period 1983–2013. Of 169 cases, 134 resected tumours were selected, for which enough tumour tissue for additional immunohistochemical analysis and adequate clinical follow-up data were available. All tumours were diagnosed, based on morphological criteria and positive reaction with at least one of the three neuroendocrine markers (CD56, chromogranin-A, and synaptophysin). All original haematoxylin and eosin stained slides were re-evaluated applying the criteria from the 2015 WHO classification, especially the number of mitoses per 2 mm² (which is about 10 high power fields, depending on the microscope), and the presence or absence of necrosis. The field of 2 mm² was defined by formula using high power field (objective 40×) and the ocular field number (18).

The fixation process was more or less consistent throughout the investigated period, due to the routinely tested pH of formalin.

Immunohistochemistry

Ki-67, cyclin A2 and cyclin B1 immunostaining was performed on formalin-fixed, paraffin embedded, 3–4 µm thick sections. Ki-67 antibody [clone MIB-1 (GA62661), ready to use, with EnVision FLEX Kit, both from Dako, Denmark] after low pH antigen retrieval was stained on Omnis (Dako). Polyclonal cyclin A2 antibody (ab80792; Abcam, United Kingdom) was stained on Dako Autostainer, after antigen retrieval in waterbath (40 min at 98°C), using EnVision detection system (Dako). Monoclonal antibody for cyclin B1 (clone Y106, ab32053, dilution 1:200; Abcam) was used on Ventana Benchmark XT with Ultraview DAB detection Kit (Ventana, USA). Slides were counterstained with haematoxylin, dehydrated and mounted.

Positive reaction was assessed as the percentage of tumour cells showing nuclear expression of Ki-67, cyclin A2, and cyclin B1. Counting was performed in the hot spot areas (areas of highest nuclear staining) after scanning the whole slide on the low power magnification, by counting of 2000 tumour cells, as previously described.²³

Statistical analysis

The significance level for statistical tests was 0.05. Homogeneity of two by two tables was tested by Fisher's exact test. Spearman's rank correlation quantified associations. Risk factors for overall survival and recurrence were analysed by Kaplan–Meier estimation and proportional hazards regression. Observation time was limited to 10 years as no deaths occurred afterwards. Median time of follow-up was calculated by the Kaplan–Meier method with the roles of censoring and event reversed.²⁴ For immunohistological staining, optimal cut-off values were calculated that minimised *p* value of the log rank tests for survival. Age was dichotomised at the median, T stage at one and N at zero. All risk factors were coded as zero or one. Therefore, the regression coefficients in Cox regression are the logarithm of the hazard ratios of two groups. As in the multivariate Cox model, many of the risk factors were strongly correlated, and the estimates had to be calculated by the elastic net method that penalises overfitting.²⁵ R 3.4.4 (www.r-project.org) and the packages *glmnet* 2.0.16 and *selectiveInference* 1.2.4 were used for calculations.

RESULTS

Clinicopathological characteristics of the study cohort

Our study cohort consisted of 97 TCs and 37 ACs. There were 72 female and 62 male patients, median age of 56 years (range 19–81 years), with median follow-up of 7.6 years [95% confidence interval (CI) 7.0–8.6]. The TC group consisted of 58 female and 39 male patients, median age of 55 years (range 19–81 years), with median follow-up of 7.6 years (95% CI 7.0–8.6). The AC group comprised 14 females and 23 males, median age of 57 years (range 22–76 years) and with median follow-up of 8.4 years (95% CI 6.7–10). TC had a median mitotic index of 1 (range 0–1.5) per 2 mm², while AC had median mitotic index of 4 (range 2–9.5). Of note, statistical analysis identified the value of 4 mitosis per 2 mm² as the ideal cut-off. When using this criterion, the group of ≤4 mitosis per 2 mm² (*n*=120) comprised all TC, but also 23 AC (23/120, 19.2%). In other words, when this ideal cut-off was applied, patients' survival curves were better separated (Fig. 1).

Diagnostic performance of Ki-67, cyclin A2 and cyclin B1

Ki-67

In the entire study cohort, the Ki-67 index was 0% in 70 (52.2%) tumours, with a range of 0–25% in the whole group.

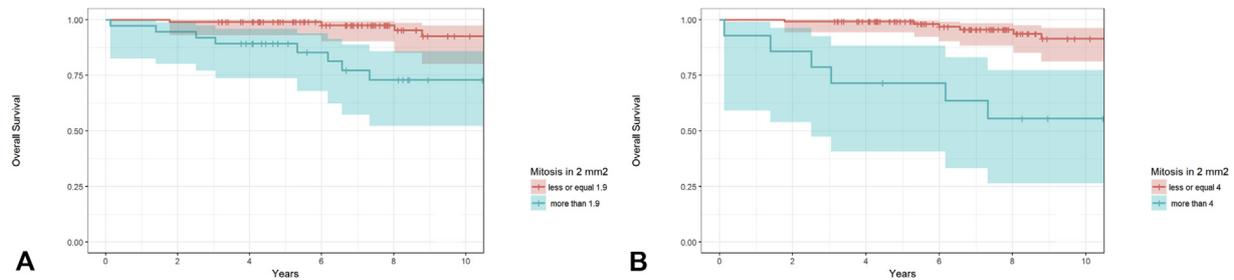


Fig. 1 (A,B) Kaplan–Meier survival curves according to the mitotic count. Better separation of patients’ survival curves is present when the cut-off of 4 mitoses (B) is applied, in comparison to the cut-off of 2 mitoses (A).

In TCs Ki-67 was 0% in 60 tumours (61.9%; median 0, range 0–15), while in the ACs it was 0% in 10 tumours (27.0%; median 5, range 0–25).

Statistical analysis calculated an ideal cut-off value of 5% for prediction of survival. In the group of Ki-67 $\leq 5\%$ TC were prevalent (92/112, 82.1%), while in the high Ki-67 group AC dominated (17/22, 77.3%), giving a significance of $p < 0.0001$ (Fisher’s exact test). Five cases diagnosed as TC according to the WHO criteria were in the high Ki-67 group. These TC had a median value of mitosis per 2 mm² of 1.5 (range 1–1.5). Furthermore, 20 cases classified as AC were in the low Ki-67 group. These had a median value of mitotic count per 2 mm² of 2.75 (range 2–8). Altogether seven cases of AC presented with a proliferation index of 20%, and three AC with a proliferation index of 25%.

Cyclin A2

All tumours together showed a median cyclin A2 value $< 1\%$ (range 0–30%). The ACs presented with a median value of 2% (range 0–30%), while tumours classified as TC showed a median value of 0% (range 0–3%). Statistical analysis detected 1% as the ideal cut-off value for prediction of survival. Applying this cut-off, in the group of $\leq 1\%$ TCs dominated (92/117, 78.6%), and in the group where cyclin A2 was $> 1\%$, ACs were predominant (12/16, 75%), giving $p < 0.0001$ (Fisher’s exact test). In other words, four cases classified as TC were in the group of high cyclin A2 expression, with a median mitotic count per 2 mm² of 1.25 (range 0.5–1.5), while 25 AC were in the low cyclin A2 group, the latter with a median mitotic count per 2 mm² of 3.5 (range 2–9.5).

Cyclin B1

In all tumour samples together, the median cyclin B1 was 1% (range 0–20). Patients with TC had a median of 1% (range 0–5), and the median in the AC group was 3% (range 0–20). An ideal cut-off value calculated by the statistical analysis was 3%. The group of $\leq 3\%$ was significantly enriched for TCs (93/120, 81.7%), while ACs predominated in the subgroup with cyclin B1 $> 3\%$ (10/14, 71.4%), resulting in a significance of $p = 0.0004$ (Fisher’s exact test). Twenty-seven cases with low ($\leq 3\%$) cyclin B1 value were classified as ACs, with a median mitotic count per 2 mm² of 3.25 (range 2–9.5). Furthermore, four cases with a high cyclin B1 value were diagnosed as TCs according to morphological criteria, demonstrating a median mitotic count per 2 mm² of 1 (range 1–1.5).

Correlations between cyclin A2, B1 and Ki-67

The correlation between cyclin A2 and cyclin B1 expression was 0.68. The correlation between cyclin A2 expression and Ki-67 proliferation was 0.37. The correlation between cyclin B1 expression and Ki-67 proliferation was 0.37. All correlations were statistically significant with $p < 0.00001$.

Survival analysis

As a group, our study cohort showed an overall survival of 87.2% (95% CI 77.9–92.8%) after 10 years of follow up, and almost as high disease-free survival (82.7%, 95% CI 73.4–89.0%). After a median follow up of 7.8 years, four TC and eight AC patients died, while eight TC and 10 AC patients had relapse. Interestingly, after 10 years all female patients were still alive, while 12 male patients had died of the disease.

Patients classified based on WHO 2015 criteria as TC had significantly better overall and disease-free survival, in comparison to AC patients ($p = 0.0045$). Furthermore, applying the ideal cut-off for cyclin A2 (1%), cyclin B1 (3%) and Ki-67 (5%), patients in the lower value groups (under the cut-off) proved also to have significantly better overall survival ($p < 0.0001$, $p < 0.0001$ and $p = 0.0006$, respectively; Fig. 2A–C), while disease-free survival was significantly better for low tumour cyclin A2 ($p < 0.0001$) and Ki-67 ($p = 0.003$), compared to patients above the cut-offs.

Univariate analysis

All results are presented in Table 1. All risk factors except age ($p = 0.06$) were statistically significant both for overall survival and relapse. The highest relative hazard was found between low and high cyclin A2, both with respect to overall survival and relapse. However, in cyclin B1 the hazard ratio was only slightly lower. The hazard ratio for the grouping according to Ki-67 was lower, but still higher than the relative risk according to ‘traditional’ diagnostic groups (TC vs AC). The relative risk of N-stage greater than 0 was between the two former risk factors.

Multivariate analysis

All results are presented in Table 1. In multivariate analysis convergence could only be achieved after regularisation with the elastic net. In a model with all risk factors, none of the risk factors was statistically significant. When we did not adjust for cyclin A2, B1 and Ki-67, some of the risk factors remained significant. The ranking of the risk factors by p

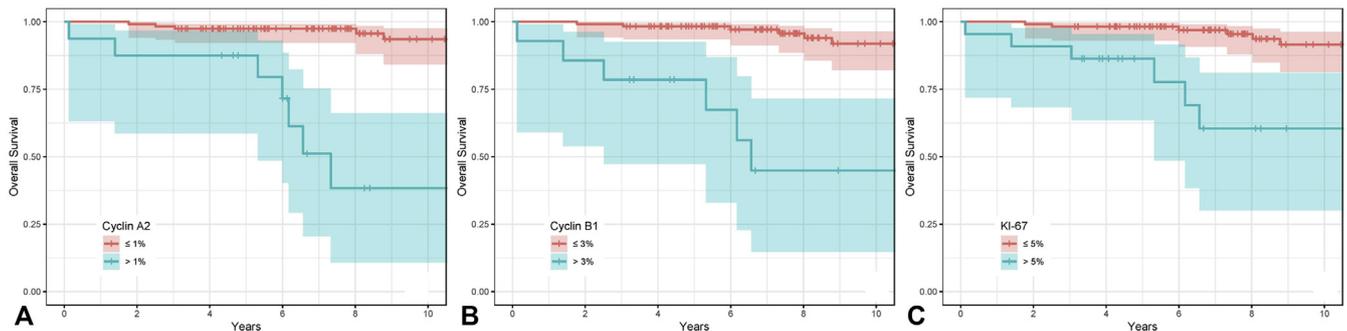


Fig. 2 Kaplan–Meier survival curves for cyclin A2, cyclin B1 and Ki-67. Separation according to the percentage of cyclin A2 (A) and cyclin B1 (B) positive cells, as well as according to the Ki-67 5% cut-off (C). All cut-off values significantly predicted patient outcome.

Table 1 Univariate and multivariate analysis of risk factors for overall survival and recurrence-free survival by Cox regression

	Univariate			Multivariate		
	HR	(95% CI)	<i>p</i>	HR	(95% CI)	<i>p</i>
Overall survival						
Gender, M	13	(1.6–98)	0.015	12	(1.8–67)	0.018
Age, >56	3.5	(0.95–13)	0.06	2.8	(0.54–9.1)	0.14
Diagnosis, AC	5.7	(1.7–19)	0.0045	4.1	(1.2–12)	0.028
T, >1	5.1	(1.1–24)	0.035	4.3	(0.81–16)	0.071
N, >0	6.3	(1.4–29)	0.018	2.6	(0.27–10)	0.24
Cyclin A2, >1%	16	(4.8–51)	0.000054	10	(2.5–>100)	0.0082
Cyclin B1, >3%	13	(4.1–40)	0.000013	6.5	(1.5–35)	0.02
Ki-67, >5%	7.4	(2.4–23)	0.00058	0.64	(0.003–5.5)	0.65
Progression-free survival						
Gender, M	6	(1.7–21)	0.0046	5.7	(1.9–16)	0.0066
Age, >56	3.1	(1.1–8.6)	0.034	3	(1.1–8)	0.042
Diagnosis, TC	3.6	(1.4–9.1)	0.007	2.5	(0.92–6)	0.064
T, >1	5.4	(1.6–19)	0.0078	5	(1.6–29)	0.015
N, >0	2.8	(1–7.4)	0.043	1.2	(0.014–2.5)	0.72
Cyclin A2, >1%	8	(3.1–21)	0.00002	3.4	(1–17)	0.049
Cyclin B1, >3%	5.8	(2.1–15)	0.00051	2.9	(0.011–7.8)	0.52
Ki-67, >5%	4.3	(1.6–11)	0.0029	0.72	(0.013–3.7)	0.65

HR refers to the category given in brackets relative to complementary category.

Multivariate analysis is adjusted for gender, age, diagnosis, T and N.

AC, atypical carcinoids; CI, confidence interval; HR, hazard ratio; M, male; TC, typical carcinoids.

value remained similar to univariate analysis. Cyclin A2 showed the highest relative hazard of all. The hazard ratio for Ki-67 was no longer significant.

DISCUSSION

Our study is the only study analysing cyclin A2 and cyclin B1 expression in carcinoid tumours of the lung, applying the same evaluation criteria as for Ki-67, and demonstrating their utility as prognostic markers.

It has been shown in previous studies that cyclin B1 overexpression correlates with poor prognosis in different carcinomas, including breast and gastric carcinomas.^{9,10} The study by Igarashi *et al.* demonstrated overexpression of cyclin B1 in the majority of pulmonary high grade NET, but in only 20% (1 case) of AC, and in none of TC.¹¹ Furthermore, they demonstrated an excellent correlation of cyclin B1 with Ki-67, stressing cyclin B1 importance in progression of NET of the lung. Furthermore, they observed a significant difference in cyclin B1 expression between TC and AC, but did not analyse this in detail. All cited studies included both cytoplasmic and nuclear staining as positive, and used a cut-off value of 15% for overexpression. Although there are

no published analyses of the cyclin A2 expression in lung NET, its roles in cell movements and epithelial to mesenchymal transition have been described.^{26,27} Namely, its downregulation increases cell motility, and downregulation and presentation in cytoplasm has been described in metastases of colon adenocarcinomas and oral squamous cell carcinomas, in comparison to primary carcinomas. In our study, for both cyclin A2 and B1 only nuclear staining was evaluated because we were primarily interested in their role in the cell cycle of carcinoids. Furthermore, cytoplasmic staining is less reliable because degraded cyclins also demonstrate positive immunohistochemical reaction. The same mode of analysis which we used for the analysis of Ki-67 proliferation index was applied. Ideal cut-off values for cyclin A2 (1%), cyclin B1 (3%) and Ki-67 (5%), determined by statistical analysis, were able to significantly predict overall survival of patients. Interestingly, in our study, all three markers demonstrated a higher relative hazard between low and high values, in comparison to the relative risk to the number of mitosis per 2 mm². In the analysis comprising all available risk factors, none was statistically significant. As the three immunostains in this study were found to be both strong risk

factors for survival and strongly correlated with each other, this behaviour was to be expected. Therefore, we excluded them from the set of risk factors. Cyclin A2 and cyclin B1 remained significant when adjusted for other risk factors, whereas Ki-67 was no longer significant. This suggests that Ki-67 is closer to conventional (clinical/histological) risk factors for survival than cyclin A2 and B1. One interesting point of the present study is that the ideal cut-off for mitotic count was identified as 4 mitoses per 2 mm². This finding is in line with some previous reports, suggesting that higher cut-off values for mitotic count could be more relevant to prognosis, and therefore for the therapy as well.^{28,29} Applying the cut-off of 4 mitoses per 2 mm² in our study cohort, we were able to stratify patients diagnosed with AC according to prognosis.

Generally, TC and AC are tumours with good prognosis, with a 10-year survival of 82–92% and 35–67%, respectively.^{30,31} A proper stratification of these rare tumours into groups with clearly different prognosis is of importance, as an adequate treatment of the intermediate-risk group (now AC) might include a wide spectrum from surgery and chemotherapy to targeted therapy. However, the present classification according to the morphological criteria is not always possible, and inter-observer reproducibility is not good enough.²¹ As an additional diagnostic criteria for lung NET, Ki-67 has been used in many different studies, with different cut-off values (ranging from 2.5 to 7.0%).^{20,21} On the other hand, Fabbri *et al.* showed that the Ki-67 labelling index is concordant regardless of the way it is determined (in full ×40 high power field, using 500, 2000 cells or 2 mm² surface), and more importantly, that results in biopsy samples correlate with the results of resection specimens.²³ However, so far the addition of Ki-67 proliferation index to histology in prediction of prognosis has been limited.^{22,32–36} Rindi *et al.* proposed a grading system for lung neuroendocrine tumours, based on Ki-67, mitosis count and necrosis, demonstrating an effective grading system.³⁷ Cyclin A2 and cyclin B1 demonstrated prognostic importance in regard to overall survival and progression-free survival in multivariate analysis, making them possible new diagnostic/prognostic markers.

We are aware of the limitations of our study. It is a retrospective study with archived material, which might influence immunohistochemical staining and analysis. It was performed on resection materials, which allowed us to appreciate heterogeneity of the staining, but at this point a correlation of small biopsy and resection material is missing.

Based on the current knowledge and available published data, it is clear that the present classification/differentiation between TC and AC is not ideal. A more sensitive grading system should improve patients' stratification, enabling optimal therapy options. We clearly demonstrated that a mitotic count of 4 per 2 mm² was the ideal cut-off point in our group, and that application of cyclin A2 and cyclin B1 might bring additional value regarding the overall and progression-free survival of TC and AC patients.

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References

- Sherr CJ. The Pezcoller lecture: cancer cell cycles revisited. *Cancer Res* 2000; 60: 3689–95.
- Choi YJ, Anders L. Signaling through cyclin D-dependent kinases. *Oncogene* 2014; 33: 1890–903.
- Beroukhim R, Mermel CH, Porter D, *et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* 2010; 463: 899–905.
- Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. *Nat Rev Cancer* 2011; 11: 558–72.
- De Boer L, Oakes V, Beamish H, *et al.* Cyclin A/cdk2 coordinates centrosomal and nuclear mitotic events. *Oncogene* 2008; 27: 4261–8.
- Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. *EMBO J* 1992; 11: 961–71.
- Bendris N, Lemmers B, Blanchard JM, Arsic N. Cyclin A2 mutagenesis analysis: a new insight into CDK activation and cellular localization requirements. *PLoS One* 2011; 6: e22879.
- Marais A, Ji Z, Child ES, *et al.* Cell cycle-dependent regulation of the forkhead transcription factor FOXK2 by CDK-cyclin complexes. *J Biol Chem* 2010; 285: 35728–39.
- Koliadi A, Nilsson C, Holmqvist M, *et al.* Cyclin B is an immunohistochemical proliferation marker which can predict for breast cancer death in low-risk node negative breast cancer. *Acta Oncol* 2010; 49: 816–20.
- Begnami MD, Fregnani JH, Nonogaki S, Soares FA. Evaluation of cell cycle protein expression in gastric cancer: cyclin B1 expression and its prognostic implication. *Hum Pathol* 2010; 41: 1120–7.
- Igarashi T, Jiang SX, Kameya T, *et al.* Divergent cyclin B1 expression and Rb/p16/cyclin D1 pathway aberrations among pulmonary neuroendocrine tumors. *Mod Pathol* 2004; 17: 1259–67.
- Suzuki T, Urano T, Miki Y, *et al.* Nuclear cyclin B1 in human breast carcinoma as a potent prognostic factor. *Cancer Sci* 2007; 98: 644–51.
- Nozoe T, Korenaga D, Kabashima A, *et al.* Significance of cyclin B1 expression as an independent prognostic indicator of patients with squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2002; 8: 817–22.
- Zheng H, Hu W, Deavers MT, *et al.* Nuclear cyclin B1 is overexpressed in low-malignant-potential ovarian tumors but not in epithelial ovarian cancer. *Am J Obstet Gynecol* 2009; 201: 367. e1–6.
- Baldin V, Lukas J, Marcote MJ, Pagano M, Draetta G. Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes Dev* 1993; 7: 812–21.
- Thomas GR, Nadiminti H, Regalado J. Molecular predictors of clinical outcome in patients with head and neck squamous cell carcinoma. *Int J Exp Pathol* 2005; 86: 347–63.
- Jin M, Inoue S, Umemura T, *et al.* Cyclin D1, p16 and retinoblastoma gene product expression as a predictor for prognosis in non-small cell lung cancer at stages I and II. *Lung Cancer* 2001; 34: 207–18.
- Travis W, Brambilla E, Burke A, Marx A, Nicholson A. *WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart*. Lyon: IARC Press, 2015.
- Caplin ME, Baudin E, Ferolla P, *et al.* Pulmonary neuroendocrine (carcinoid) tumors: European Neuroendocrine Tumor Society expert consensus and recommendations for best practice for typical and atypical pulmonary carcinoids. *Ann Oncol* 2015; 26: 1604–20.
- Pelosi G, Rindi G, Travis WD, Papotti M. Ki-67 antigen in lung neuroendocrine tumors: unraveling a role in clinical practice. *J Thorac Oncol* 2014; 9: 273–84.
- Liu SZ, Staats PN, Goicochea L, *et al.* Automated quantification of Ki-67 proliferative index of excised neuroendocrine tumors of the lung. *Diagn Pathol* 2014; 9: 174.
- Swartz DR, van Suylen RJ, den Bakker MA, *et al.* Interobserver variability for the WHO classification of pulmonary carcinoids. *Am J Surg Pathol* 2014; 38: 1429–36.
- Fabbri A, Cossa M, Sonzogni A, *et al.* Ki-67 labeling index of neuroendocrine tumors of the lung has a high level of correspondence between biopsy samples and surgical specimens when strict counting guidelines are applied. *Virchows Arch* 2017; 470: 153–64.
- Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. *Control Clin Trials* 1996; 17: 343–6.
- Simon N, Friedman J, Hastie T, Tibshirani R. Regularization paths for Cox's proportional hazards model via coordinate descent. *J Stat Softw* 2011; 39: 1–13.
- Arsic N, Bendris N, Peter M, *et al.* A novel function for Cyclin A2: control of cell invasion via RhoA signaling. *J Cell Biol* 2012; 196: 147–62.

27. Wang YF, Chen JY, Chang SY, *et al.* Nm23-H1 expression of metastatic tumors in the lymph nodes is a prognostic indicator of oral squamous cell carcinoma. *Int J Cancer* 2008; 122: 377–86.
28. Klemen H, Smolle-Jüttner FM, Popper HH. Morphological and immunohistochemical study of typical and atypical carcinoids of the lung, on the bases of 55 cases with clinico-pathological correlation and proposal of a new classification. *Endocr Relat Cancer* 1994; 1: 53–62.
29. Beasley MB, Thunnissen FB, Brambilla E, *et al.* Pulmonary atypical carcinoid: predictors of survival in 106 cases. *Hum Pathol* 2000; 31: 1255–65.
30. García-Yuste M, Matilla JM, Cueto A, *et al.* Typical and atypical carcinoid tumours: analysis of the experience of the Spanish multicentric study of neuroendocrine tumours of the lung. *Eur J Cardiothorac Surg* 2007; 31: 192–7.
31. Fink G, Krelbaum T, Yellin A, *et al.* Pulmonary carcinoid: presentation, diagnosis, and outcome in 142 cases in Israel and review of 640 cases from the literature. *Chest* 2001; 119: 1647–51.
32. de Vilhena AF, das Neves Pereira JC, Parra ER, *et al.* Histomorphometric evaluation of the Ki-67 proliferation rate and CD34 microvascular and D2-40 lymphovascular densities drives the pulmonary typical carcinoid outcome. *Hum Pathol* 2018; 81: 201–10.
33. Clay V, Papaxoinis G, Sanderson B, *et al.* Evaluation of diagnostic and prognostic significance of Ki-67 index in pulmonary carcinoid tumours. *Clin Transl Oncol* 2017; 19: 579–86.
34. Joseph MG, Shibani A, Panjwani N, *et al.* Usefulness of Ki-67, mitoses, and tumor size for predicting metastasis in carcinoid tumors of the lung: a study of 48 cases at a tertiary care centre in Canada. *Lung Cancer Int* 2015; 2015: 545601.
35. Swarts DR, Rudelius M, Claessen SM, *et al.* Limited additive value of the Ki-67 proliferative index on patient survival in World Health Organization-classified pulmonary carcinoids. *Histopathology* 2017; 70: 412–22.
36. Garg R, Bal A, DAS A, Singh N, Singh H. Proliferation marker (Ki67) in sub-categorization of neuroendocrine tumours of the lung. *Turk Patoloji Derg* 2019; 35: 15–21.
37. Rindi G, Klersy C, Inzani F, *et al.* Grading the neuroendocrine tumors of the lung: an evidence-based proposal. *Endocr Relat Cancer* 2013; 21: 1–16.