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JOANA SOFIA ANDRÉ MONTEIRO

mTOR AND ERCC1 – IMMUNOHISTOCHEMISTRY EXPRESSION IN SMALL CELL LUNG CARCINOMA

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TRABALHO REALIZADO SOB A ORIENTAÇÃO DE:
PROFESSORA DOUTORA LINA CARVALHO
DRA. ANA ALARCÃO
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Abstract

Introduction: Lung cancer is a leading cause of death in most industrialized countries. Without treatment, small cell lung carcinoma is considered the most aggressive of the lung tumors with a median survival ranging from 2 months to 4 months. The long-term prognosis for patients with small cell lung carcinoma is relatively poor, and only 5% to 10% are expected to live for at least 5 years after diagnosis.

Material and Methods: A total of 39 patients (7 women, 32 men) with small cell lung cancer diagnosed between 1998 and 2001.

Results: No relation between survival and clinical parameters (gender, age and stage of disease) was demonstrated in this study. Patients expressing positive mTOR had significantly longer survival (log-rank p = 0,017). Expression from ERCC1 was not correlated with survival (log-rank p= 0,808). Patients with positive mTOR or negative ERCC1 immunohistochemistry had longer survival when treated with carboplatin + etoposide (respectively p = 0,02 and p = 0,001)

Conclusion: This data indicates the possible prognostic value for mTOR in patients diagnosed with small cell lung carcinoma. ERCC1 lower expression and mTOR overexpression are associated to a better response when applied first line chemotherapy.

Keywords: Small cell lung cancer, immunohistochemistry, mTOR, ERCC1, etoposide, carboplatin.

Introduction

Lung cancer is a leading cause of death in most industrialized countries [1] and approximately 85% of these cases are adenocarcinomas, epidermic carcinomas and other histological types with (15%) comprising small cell lung carcinomas (SCLC) [2] Small cell lung carcinoma, large cell neuroendocrine carcinoma (LCNEC), typical carcinoid (TC), and atypical carcinoid (AC) form a rough spectrum of lung carcinomas with neuroendocrine features (NE). Very different when regarding the natural course of the tumors and treatment strategies.

In 98% of cases correlated with tobacco smoking and patients have a very poor outcome with a 5-year survival lower than 5% [3]

Without treatment, SCLC is considered the most aggressive of the lung tumors with a median survival ranging from 2 months to 4 months [3]. The long-term prognosis for patients with SCLC is relatively poor, and only 5% to 10% are expected to live for at least 5 years after diagnosis. [4-6]

At the time of diagnosis, approximately 30% of patients with SCLC will have a tumor that is confined to the following areas: hemithorax of origin, the mediastinum, or the supraclavicular lymph nodes [7]. These patients have limited disease and can be encompassed within reasonable radiotherapy fields as limited-stage disease SCLC (LD-SCLC). With currently available treatments, the median survival for patients with LD-SCLC ranges from 16 months to 26 months [8-10]. Patients with tumors that have metastasized beyond supraclavivular areas have extensive-stage disease ED-SCLC and with currently available treatment options, the median survival varies from 6 months to

12 months. [11] The combination of platinum compound (cisplatin or carboplatin) with etoposide is currently considered the standard of care for both extensive and limited disease, and in the latter usually in combination with concurrent or sequential thoracic radiotherapy. [12]

Early recurrence and subsequent resistance to therapy is the main cause of poor outcome in these patients and determination of who would benefit from therapy and who would not, has potential clinical implications. [13]

Etoposid, a topoisomerase II inhibitor has a significant activity on a large range of carcinomas, in particular on SCLC, germ cell tumors, and haematologic and childhood malignancies. [14] Its cytotoxicity is schedule dependent. [15]

The way that cisplatin operates is by forming a platinum complex inside of a cell which binds to DNA and cross-links DNA. When DNA is cross-linked in this manner, it causes the cells to undergo apoptosis, or systematic cell death. One of the methods it uses causes apoptosis through cross-linking is by damaging the DNA so that the repair mechanisms for DNA are activated, and once the repair mechanisms are activated and the cells are found to not be salvageable, the death of those cells is triggered instead.

Principal enzyme present in NER (nucleotide excision repair) pathway. It is codified by ERCC1 gene, 15 kb, present in the 19q chromosome. NER is responsible for 2 actions: repair lesions in the genome – global genome NER - and repair blocking transcription lesions in DNA – transcription-couple NER. That is way NER can play an important role in carcinogenesis and in treatment response, the improvement in repair DNA ability leads to resistance to chemotherapy drugs. [16]. Several studies indicate that

low ERCC1 is correlated with better chemotherapy response but lower overall survival. [16,17]

The PI3K/Akt/mTOR pathway and its implication in human cancer have been extensively reviewed in the past years [18,19,20]. mTOR has been identified as a downstream target of both the PI3K [21,22] and Ras [23-26] signaling pathways. The discovery of the highly specific and potent mTOR inhibitor rapamycin and its derivatives (RAD001, CCI-779, and AP23573) further boosted the interest of the scientific community in elucidating its biological function [27,28].

Inhibition of the PI3K/Akt pathway was shown to reverse the mitogenic effects of these autocrine loops [29]. In addition, immunohistochemical analysis detected high levels (70%) of phosphorylated Akt in tumor tissues from SCLC patients and revealed the implication of the activated pathway in disease progression [30,31].

Further studies have shown that inhibition of the PI3K/Akt/mTOR pathway with LY294002 or rapamycin led to apoptosis and decreased cell growth in SCLC cells [32].

mTOR is present in two distinct protein complexes commonly referred to as mTOR complex 1 (mTORC1) andmTOR complex 2 (mTORC2. mTORC1 and mTORC2 phosphorylate different substrates to regulate distinct cellular functions. For instance, mTORC2 phosphorylates AKT, SGK1 and PKC (members of the AGC kinase family) which control cell survival and cytoskeletal organization [33-36]. mTORC1, on the other hand, stimulates cell growth and proliferation by increasing cap-dependent translation initiation and this is mediated by its two major downstreamtargets: the eIF4E-binding proteins (4EBPs) and S6 kinases (S6K1 and S6K2). mTORC1 signalling is frequently dysregulated in cancer [33,37]. Loss or inactivation of tumor suppressors such as p53,

LKB1, TEN, and TSC1/2, which antagonize PI3K-dependent activation of mTORC1, can promote tumorigenesis via increased signalling through mTORC1 [38-41]. Moreover, increased levels and/or phosphorylation of downstream targets of mTORC1 have been reported in various human malignancies in which they correlate with tumor aggressiveness and poor prognosis [42]. S6K1 is reported to be overexpressed in breast cancer [43] and 4E-BP1 is downregulated and/or hyperphosphorylated (i.e. inactivated) in breast, ovarian, and other cancers [44-46].

Known interactions between mTOR pathway and other signaling pathways, the synergistic antitumor effect of mTOR inhibitors and anti-angiogenic agents is under evaluation in clinical trials. The mTOR inhibitors will likely be most effective when combined with traditional chemotherapy. Clinical trials evaluate the safety and efficacy of mTOR inhibitors with concomitant administration of 5-fluoruracil/leucovorin in patients with refractory colorectal cancer. Stomatitis was the dose limiting toxicity while no pharmakokinetic synergism between mTOR inhibitors and chemotherapy was observed. [47]

Taken together, these findings link aberrant mTORC1 signalling with dysregulated translational control in cancer. As a result, mTORC1 has emerged as an important target for anti-cancer therapy. [48]

Thus, we hypothesize that effective therapy of SCLC tumors will require combinations of targeted drugs with anti-angiogenic properties in combination with mTOR inhibitors, in order to inhibit a large panel of SCLC molecular dysfunctions and to prevent the development of acquired secondary resistance.

The present piece of work was formulated to be applied to small biopsies of SCLC, where tumor tissue amount is reduced and mainly devoted to diagnosis, with lymphoma as the first differencial diagnosis. By applying ERCC1 (clone) and mTOR (clone), it was postulated wether ERCC1 score would be related to survival or/and to tumor size reduction as well as tumoral cells expression of mTOR, to understand different populations of SCLC.

Material and methods

Human Subject

Patients had SCLC diagnosed at Coimbra University Hospital between January 1998 and December 2001. A total of 39 cases (7 women, 32 men) were selected for this study according with available formalin-fixed paraffin-embedded tissue from routine histopathological examination before chemotherapy.

Histological diagnosis was made according to the World Health Organization guidelines. The following clinical variables were registered: age at diagnosis time, stage of disease (ED-SCLC and LD-SCLC) and survival. Limited stage disease is defined as disease confined to one hemithorax and adjacent nodes (Table I summarizes the studied cases).

Chemotherapy treatments were identified from medical records, standard first line treatment was etoposide and carboplatin and second line treatment, a triplet consisting on ciclofosfamine, etoposide and epirubicin.

The survival time was defined as the interval in days from histological diagnosis till death.

Patient	Age	Gender	Stage
1	54	M	ED-SCLC
2	61	M	ED-SCLC
3	71	M	ED-SCLC
4	74	M	LD-SCLC
5	53	M	LD-SCLC
6	74	M	LD-SCLC
7	56	M	ED-SCLC
8	59	M	ED-SCLC
9	74	M	ED-SCLC
10	70	F	ED-SCLC
11	76	M	ED-SCLC
12	66	M	ED-SCLC
13	66	F	ED-SCLC
14	83	M	ED-SCLC
15	62	F	LD-SCLC
16	79	M	ED-SCLC
17	57	M	ED-SCLC
18	54	F	ED-SCLC
19	69	M	LD-SCLC
20	78	M	LD-SCLC
21	87	F	ED-SCLC
22	64	M	ED-SCLC
23	80	M	ED-SCLC
24	57	M	ED-SCLC
25	77	M	ED-SCLC
26	70	M	LD-SCLC
27	73	M	ED-SCLC
28	49	M	ED-SCLC
29	62	M	LD-SCLC
30	67	M	ED-SCLC
31	69	M	LD-SCLC
32	68	F	LD-SCLC
33	69	M	LD-SCLC
34	49	M	LD-SCLC
35	74	F	LD-SCLC
36	63	M	ED-SCLC
37	47	M	ED-SCLC
38	59	M	ED-SCLC
39	78	M	ED-SCLC

Table I. Studied population. M – male; F- Female.

Tissue Analysis - Immunohistochemistry

The immunohistochemical study was performed on formalin-fixed, paraffin-embedded lung tissue samples as said before and the sections were placed on sialynized slides (three-micrometer tissue sections) and allowed to dry overnight.

Immunohistochemical staining was performed with Bond Polymer Refine Detection™ to Bond-Max auto-stainer (DS9800; Novocastra Laboratories Ltd, Newcastle, United Kingdom) to apply ERCC1 Ab-2 (8F1, Thermo Fisher Scientific, Fremont, CA, USA) and mTOR (Ser2448) (N/A, Imgenex, San Diego, CA, USA) according to manufacturer's instructions.

In parallel, known positive and negative controls were used tonsil sample as positive control for ERCC1 and a section of a ductal carcinoma of the breast as positive control for mTOR.

Each immunohistochemical antibody was scored according with four variables: negative, +, ++ and +++ corresponding respectively to 0%; till 50%, between 50 and 75% and over 75 cells expressing the antibody.

Statistical Analysis

The statistical analysis was performed with SPSS Version17.0. Lifetable probabilities of overall survival were performed by the Kaplan–Meier method (Kaplan and Meier, 1958), and differences in survival between subgroups of patients were compared with the log-rank test (Mantel, 1996). A P-value < 0.05 was considered significant.

Results

Patient	ERCC1	mTOR	Cromogranin	Chemotherapy	Survival (days)
1	neg	neg	neg	no chemotherapy	181
2	neg	N +++	pos +++	etoposide+carboplatin	120
3	neg	N +++	pos +++	no chemotherapy	117
4	N++	N +++	neg	etoposide+epirubicin+cyclophophamide	258
5	N++	N+	neg	etoposide+epirubicin+cyclophophamide	50
6	neg	N+++	neg	etoposide+epirubicin+cyclophophamide	101
7	N++	N++	neg	etoposide+epirubicin+cyclophophamide	399
8	N++	neg	pos +++	etoposide+carboplatin	59
9	N+	N+++	pos ++	etoposide+carboplatin	73
10	N+	N++	pos ++	etoposide+carboplatin	428
11	neg	N+++	pos ++	etoposide+carboplatin	481
12	N++	neg	pos ++	etoposide+carboplatin	63
13	neg	N+++	pos +	etoposide+epirubicin+cyclophophamide	110
14	N+++	N++	pos +	etoposide+carboplatin	137
15	N+	N+++	pos +++	etoposide+epirubicin+cyclophophamide	71
16	N+++	N++	pos ++	no chemotherapy	135
17	neg	neg	neg	no chemotherapy	6
18	N++	neg	pos +++	etoposide+carboplatin	61
19	N+++	neg	neg	etoposide+carboplatin	291
20	N+	N++	neg	etoposide+carboplatin	752
21	N++	neg	pos +++	no chemotherapy	3
22	N++	N+++	neg	etoposide+epirubicin+cyclophophamide	184
23	N++	N+++	pos +++	etoposide+carboplatin	264
24	N++	N+++	pos +	etoposide+carboplatin	356
25	neg	neg	pos +	etoposide+carboplatin	170
26	neg	neg	pos ++	no chemotherapy	166
27	N+++	N+++	pos +++	etoposide+carboplatin	118
28	neg	neg	neg	etoposide+carboplatin	184
29	N+++	N+++	pos ++	etoposide+carboplatin	389
30	neg	neg	neg	etoposide+carboplatin	180
31	N++	N++	neg	etoposide+carboplatin	411
32	neg	N+++	pos ++	etoposide+carboplatin	485
33	N+++	N++	pos +	no chemotherapy	146
34	N+++	N++	neg	etoposide+carboplatin	138
35	N++	neg	pos +	no chemotherapy	53
36	N++	neg	neg	etoposide+carboplatin	281
37	neg	N+++	pos +++	etoposide+carboplatin	472
38	neg	N+++	pos +	no chemotherapy	18
39	N++	neg	pos ++	etoposide+carboplatin	68

Table II. Immunohistochemistry, chemotherapy treatments and survival in studied cases.

Clinical parameters

The mean age at the time of diagnosis of the 7 women (17,9%) and 32 men was 66,62 years. In total, 23 (59%) patients were aged > 65 years.

A total of 13 patients initially presented with limited disease, while 26 patients were found to have extensive disease.

At the time of these analysis the patients were already dead. The overall mean survival was 205 days (6,8 months), and median was 146 days (4,9 months); 8 (20,5%) patients remained alive after one year. (Table III, Fig.1, Fig. 3).

Means and Medians for Survival Time

Mean[a]				Median			
		95% Confidence Interval				95% Confidence Interval	
Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
204.590	26.927	151.812	257.367	146.000	21.850	103.173	188.827

a. Estimation is limited to the largest survival time if it is censored.

Table III– Means and Medians for Survival

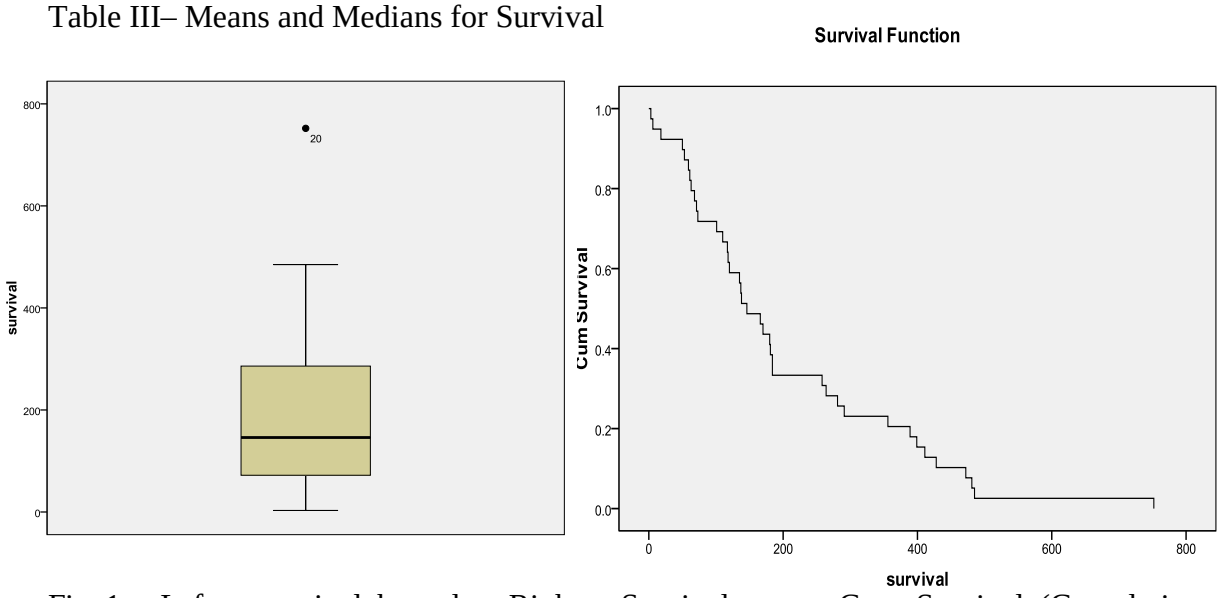

Fig 1 . Left – survival box-plot. Right - Survival curve. Cum Survival (Cumulative Survival – Percentage). Survival by days.

No relation between survival and clinical parameters (gender, age and stage of disease) was demonstrated in this study.

The mean survival for women was 173 days (5,8 months, with a 95% confidence interval from 1 month to 10,6 months) and 212 days for men (7 months, with a 95% confidence interval from 5,2 months to 8,6 months), log-rank $p = 0{,}810$.

Mean survival for patients older than 65 years was 217,826 days (7,2 months, with a 95% confidence interval from 4,8 months to 9,7 months) and 185,563 days for the other patients (6,2 months, with a 95% confidence interval from 3,7 months to 8,6 months), log-rank $p = 0{,}458$.

The mean survival for LD-SCLC was 255 days (8,5months, with a 95% confidence interval from 4,7 months to 12,2 months) and 179 days for ED-SCLC (6 months, with a 95% confidence interval from 4,1 months to 7,8 months), log-rank $p = 0{,}208$.

Survival Means and range			
		95% Confidence Interval (days)	
	Mean	Lower Bound	Upper Bound
Gender (Female vs Male)			
Female	173.000	27.144	318.856
Male	211.500	154.688	268.312
Age (<65 & >65)			
<65 years	185,563	112,107	259,018
>65 years	217,826	143,500	292,152
Extension			
LD-SCLC	254.692	141.815	367.569
ED-SCLC	179.538	124.733	234.344

Table IV – Survival means and Confidence Intervals (days)

Chemotherapy Treatment

A total of 30 (76,9%) patients received chemotherapy during the study period: 23 (77%) received first line chemotherapy etoposide + carboplatine and 7 (23%) received second line chemotherapy regimen which included etoposide + epirubicin + cyclophosphamide. (fig. 8)

Means of survival according to the two different chemotherapy protocols and the comparison of those means are described respectively in tables V and VI.

Means and Medians for Survival Time

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
protocols	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
etoposide+carboplatine	260.043	38.383	184.813	335.274	184.000	75.064	36.875	331.125
etoposide+epirubicin+cyclophosphamide	167.571	47.031	75.390	259.753	110.000	11.784	86.904	133.096
no chemotherapy	91.667	23.892	44.838	138.495	117.000	95.406	.000	303.995
Overall	204.590	26.927	151.812	257.367	146.000	21.850	103.173	188.827

a. Estimation is limited to the largest survival time if it is censored.

Table V – Means and medians for survival time according to chemotherapy treatment.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	5.328	2	.070

Test of equality of survival distributions for the different levels of protocols.

Table VI – Test of equality – chemotherapy treatments

The test of equality of survival distributions for the different chemotherapy protocols was not significantly different, log-rank p = 0,070.

Immunohistochemistry

The distribution of mTOR and ERCC1 in the 39 patients is showed on Table VII.

mTORgroup * ERCC1group Crosstabulation

Count

		ERCC1group		
		negative	positive	Total
mTORgroup	Negative	6	8	14
	positive	8	17	25
Total		14	25	39

Table VII. mTOR and ERCC1 expression.

mTOR

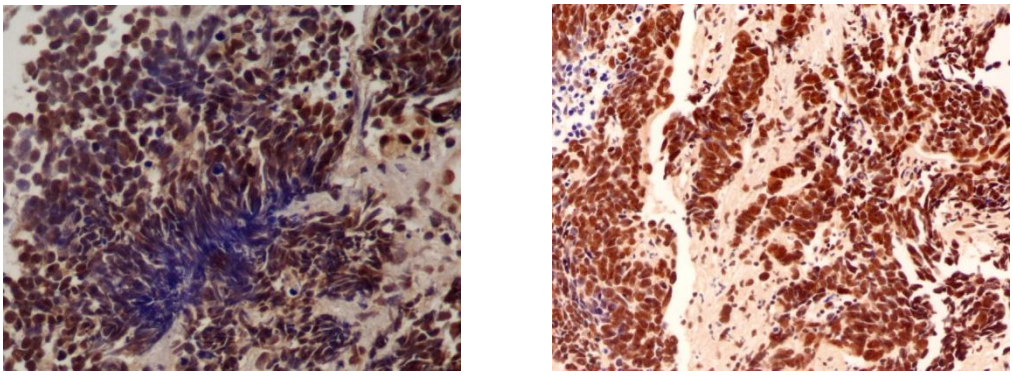

Fig 2. mTOR x 200. Nuclear staining.

Means of survival in the two different groups, expression positive and negative mTOR antibody are described in table VIII. Test of equality of survival according to mTOR groups and survival curves are represented in table IX and fig. 5.

Means and Medians for Survival Time

	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
mTORgroup	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
negativo	126.143	25.137	76.875	175.410	68.000	96.348	.000	256.841
positivo	248.520	37.085	175.834	321.206	146.000	39.135	69.295	222.705
Overall	204.590	26.927	151.812	257.367	146.000	21.850	103.173	188.827

a. Estimation is limited to the largest survival time if it is censored.

Table VIII. Means and Medians - population expressing mTOR

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	5.683	1	.017

Test of equality of survival distributions for the different levels of mTORgroup.

Table IX. test of equality of survival –mTOR positive and negative

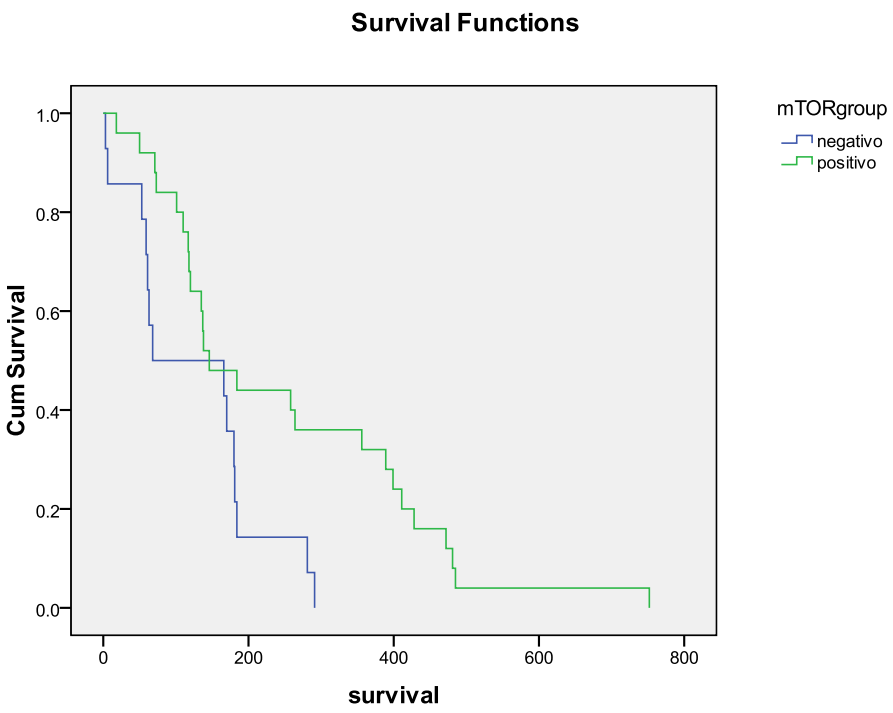

Fig. 3 – Survival curves – mTOR positive and negative.

In this study patients expressing positive mTOR had significantly longer survival (log-rank p = 0,017) than patients who did not express this antibody.

ERCC1

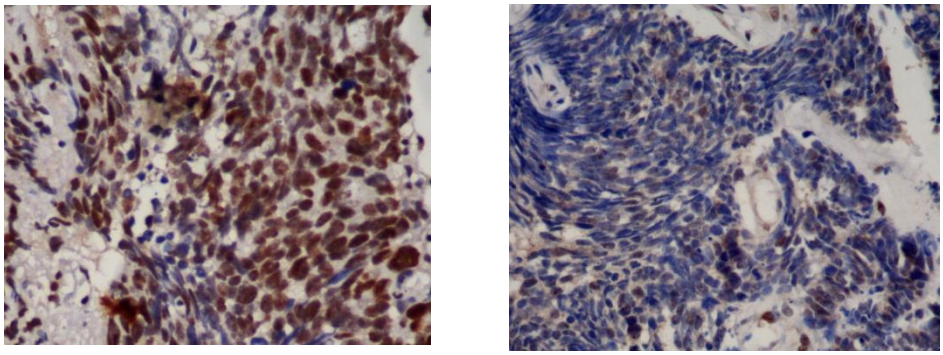

Fig. 4. ERCC1 x 400. Nuclear staining.

The mean survival for patients who express positive ERCC1 was 207,520 days (6,9 months with a 95% confidence interval from 4,6 months to 9,2 months) and 199, 357 days (6,6 months with a 95% confidence interval from 3,8 months to 8 months) for those scored negative. From the test of equality of survival distribution in this two groups no significant difference in means survival was confirmed, log-rank p=0,808.

Chemotherapy

mTOR

Patients who expressed negative mTOR: 9 were treated with carboplatin+etoposid and 5 were not submitted to chemotherapy (table X). It was not possible to compare means of survival for different protocols in this population.

Case Processing Summary

protocols	Total N	N of Events	Censored	
			N	Percent
etoposide+carboplatin	9	9	0	.0%
no chemotherapy	5	0	5	100.0%
Overall	14	9	5	35.7%

Table X. Chemotherapy treatments in patients expressing mTOR negative.

In patients scored positive for mTOR (25, 64%) 14 received first line chemotherapy, 7 second-line treatment and 4 were not submitted to chemotherapy (table XI describes this distribution).

Case Processing Summary

protocols	Total N	N of Events	Censored	
			N	Percent
etoposide+carboplatine	14	14	0	.0%
etoposide+epirubicin+cyclophosphamide	7	7	0	.0%
no chemotherapy	4	0	4	100.0%
Overall	25	21	4	16.0%

Table XI. patients mTOR positive – different chemotherapy treatments.

In this population comparison between survival means for the different chemotherapy protocols (table XII) reveal they were significantly different, log rank p = 0,02. Patients treated with etoposide + carboplatin lived longer, mean survival 330,286 (11 months), than patients who received the triplet second line treatment, mean survival for this group 167,571 days (5,6 months). Table XIII summarizes the means and confidence interval for this group.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	7.848	2	.020

Test of equality of survival distributions for the different levels of protocols.

Table XII. patients mTOR positive. Test of equality of survival means for different chemotherapy treatments

Means and Medians for Survival Time

protocols	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
etoposide+carboplatine	330.286	52.360	227.661	432.910	356.000	116.927	126.823	585.177
etoposide+epirubicin+cyclophosphamide	167.571	47.031	75.390	259.753	110.000	11.784	86.904	133.096
no chemotherapy	104.000	29.283	46.605	161.395	117.000	58.500	2.340	231.660
Overall	248.520	37.085	175.834	321.206	146.000	39.135	69.295	222.705

a. Estimation is limited to the largest survival time if it is censored.

Table XIII. patients mTOR positive - means and medians of survival according to chemotherapy treatments.

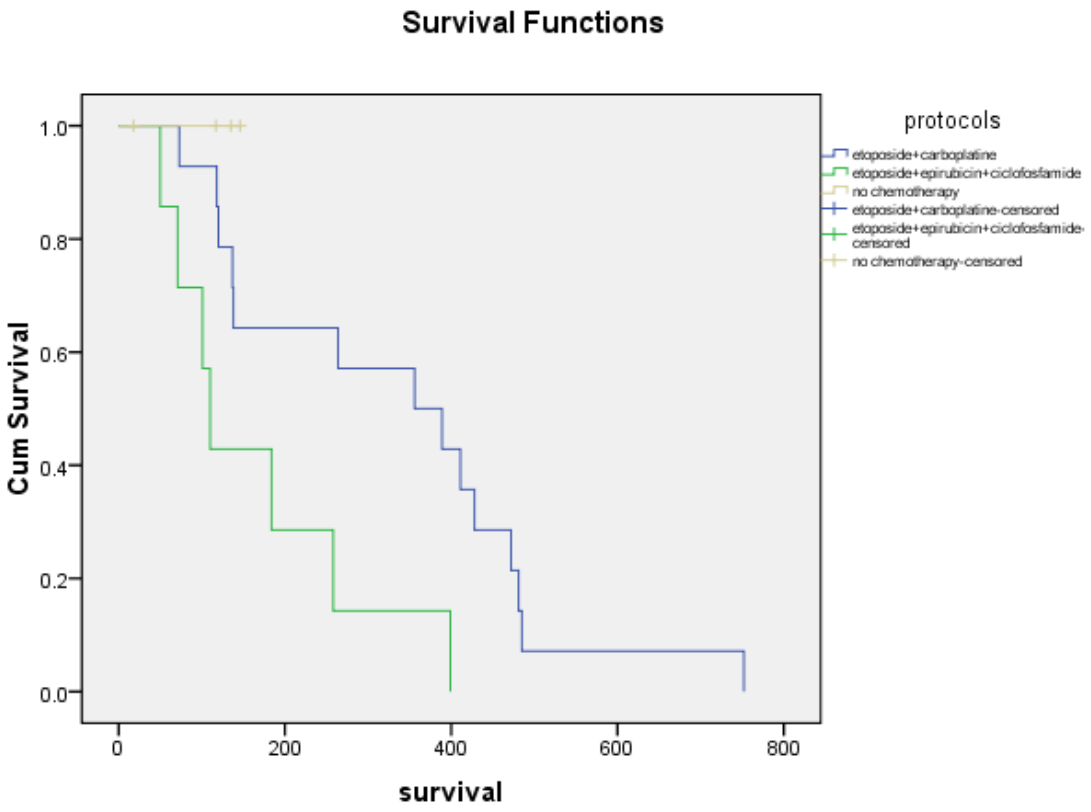

Fig. 5. mTOR positive – survival curve according to chemotherapy treatments

ERCC1

In this study, 14 patients did not express ERCC1 in SCLC cells, in this group 7 were submitted to first line chemotherapy, 2 were treated with etoposide+epirubicin+cyclophosphamide and 5 did not received this type of treatment, this distribution is indicated in table XIV.

Case Processing Summary

Protocols	Total N	N of Events	Censored	
			N	Percent
etoposide+carboplatine	7	7	0	.0%
etoposide+epirubicin+cyclophosphamide	2	2	0	.0%
no chemotherapy	5	0	5	100.0%
Overall	14	9	5	35.7%

Table XIV. ERCC1 negative -distribution of patients according to chemotherapy protocol

Patients treated with first line chemotherapy had a mean survival of 298,857 days (10 months) and those who received second line therapy had a mean survival of 105,500 days (3,51 months). Table XV show means and medians for survival time according to chemotherapy treatment.

This difference in survival time was analyzed using Kaplan-meier method and it was significantly different, log-rank p = 0,001. (table XVI)

Means and Medians for Survival Time								
	Mean[a]				Median			
			95% Confidence Interval				95% Confidence Interval	
protocols	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
etoposide+carboplatine	298.857	64.313	172.804	424.910	184.000	5.237	173.735	194.265
etoposide+epirubicin+cyclophophamide	105.500	4.500	96.680	114.320	101.000	.	.	.
no chemotherapy	97.600	36.563	25.936	169.264	117.000	108.449	.000	329.560
Overall	199.357	43.170	114.744	283.971	166.000	46.771	74.329	257.671
a. Estimation is limited to the largest survival time if it is censored.								

Table XV – ERCC1 negative – means and medians according to chemotherapy.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	14.058	2	.001

Test of equality of survival distributions for the different levels of protocols.

Table XVI – ERCC1 negative - test of equality of survival means.

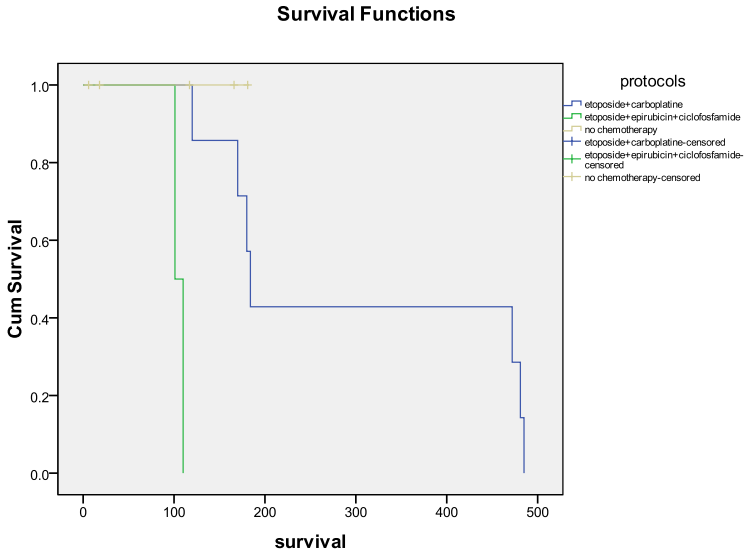

Fig 6. ERCC1 negative – survival curves

Immunohistochemistry staining was positive for ERCC1 in 25 patients: 16 received first line chemotherapy treatment, 5 were treated with second line treatment and 4 were not submitted to chemotherapy (table XVII). No relation was found between chemotherapy treatments and survival in patients expressing positive ERCC1, log-rank p = 0,420 (table XVIII), figure 7 show survival curves from these different groups.

Case Processing Summary

protocols	Total N	N of Events	Censored	
			N	Percent
etoposide+carboplatin	16	16	0	.0%
etoposide+epirubicin+cyclophosphamide	5	5	0	.0%
no chemotherapy	4	0	4	100.0%
Overall	25	21	4	16.0%

Table XVII – ERCC1 positive - chemotherapy treatments.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	1.736	2	.420

Test of equality of survival distributions for the different levels of protocols.

Table XVIII – ERCC1 positive – test of equality of survival means according to chemotherapy protocols

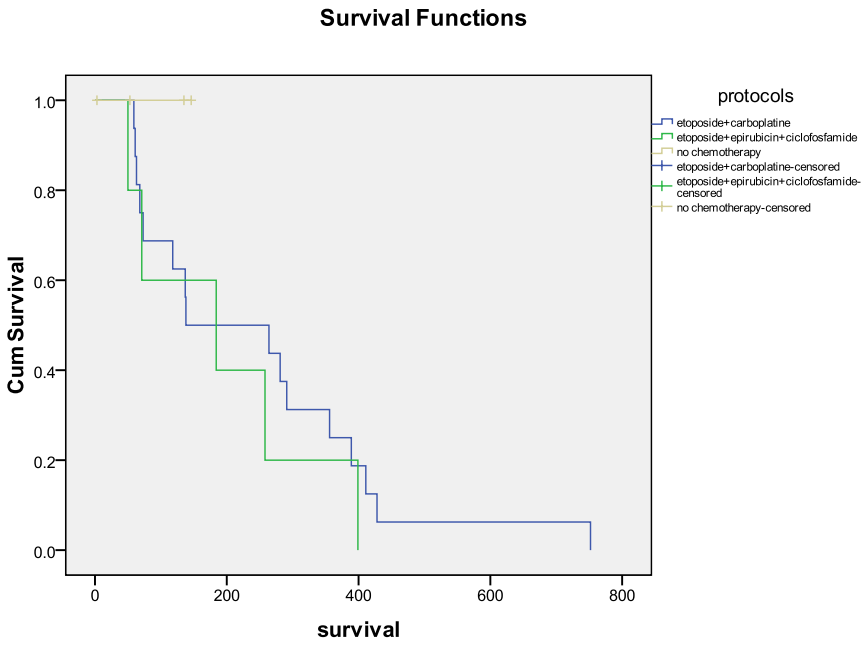

Fig 7. ERCC1 positive - Survival curves according to chemotherapy treatment

Discussion

Small cell lung cancer accounts about 15% of all cases of lung cancer, it is characterized by rapid growth and early extrathoracic spread and cytotoxic chemotherapy is the cornerstone of any therapeutic strategy. [12]

At the time of diagnosis, more than two-third of patients with SCLC are ineligible for concurrent chemoradiation therapy due to extensive-stage disease or poor-prognosis, limited-staged disease. [49]

In this study clinical parameters analyzed (age, gender and stage of disease) had no significant correlation with the overall survival.

Combination therapy represents the mainstay of treatment for this major subset of patients, and the association of etoposid with a platinum compound remains the standard

of care, offering a median survival of 9-12 months with 2 year survival rates usually < 10% [50].

Most patients with SCLC will not only improve by a reduction in tumor size, but benefit in clinical aspects, especially dyspnoea and cough. [52].

In the studied cases two major chemotherapy treatments were applied and despite no statistic difference found (log-rank p = 0,07) 3 months was the difference between survival means.

mTOR is a serine/threonine kinase which has emerged in the past 5 years as one of the most important intracellular signaling enzyme regulating cell growth, survival and motility in cancer cells.

In this study patients with mTOR scored positive had significantly longer survival, log-rank p= 0,017. This data indicates the possible prognostic value for mTOR in patients diagnosed with SCLC and identifies one possible chemotherapy target.

TOR is a serine/threonine kinase with a molecular weight of 289 kDa. TOR kinases are highly conserved and identical in up to 60% in humans and other mammalian organisms. mTOR is found in the cellular cytoplasm as a complex with other molecules. There are two distinct mTOR complexes: mTOR complex 1 (mTORC1) and mTORC2. The former is composed of the proteins mTOR, raptor (regulatory associated protein of mTOR), PRAS40 (proline-rich AKT substrate 40 kDa) and the protein mLST8/GbL.13–15 The mTORC2 complex is composed of mTOR, rictor (rapamycin insensitive companion of mTOR), mSIN1 (mammalian stress-activated protein kinase interacting protein 1), protor-1 and mLST8/GbL.14,16–18. Main functions of mTORC1 include protein synthesis and cell cycle progression whereas mTORC2 plays important role in

acting cytoskeleton organization and cell survival. These actions are executed subject to nutrient, amino acids and surface growth factors status. Of the two complexes only mTORC1 is inhibited by rapamycin, although recent data suggested that prolonged treatment with rapamycin may affect the mTORC2 assembly and Akt signaling. The mTOR intracellular pathway is activated by nutrients, mitogens, growth factors and other extracellular molecules when they interact with the outer side of the cellular membrane. Nutrients such as amino acids, glucose and oxygen, enter the cytoplasm by passive diffusion through membrane pores and activate mTOR complex 1 (mTORC1) either directly or through inhibition of the tuberous sclerosis complex 1/2. All along the PI3K/Akt/mTOR pathway we come across to deregulations of the involved molecules causing or relating to various diseases and especially carcinogenesis. Conditional on their normal function of these molecules, mutations, persistent activation or silencing may be required for the development of a disease. As a general rule, tumor growth requires amplification and overactivation of proto-oncogenes and silencing or loss of function of tumor suppressor genes. In the mTOR transduction pathway proto-oncogenes recognised so far include Ras, PI3K, Akt, Rheb, S6K1, eIF4E and Cyclin D1. Hyperactivation or overexpression of any of these genes has been demonstrated in various solid tumor types and hematological malignancies. On the other hand tumor suppressor genes involved normally in the mTOR pathway are PTEN, TSC1/2, LKB136 REDD1, p5337 and beclin138. Other important genes participating in the TOR, but not exclusively, pathway are the EGFR, IGFR and IRS genes. Downstream effectors of overexpressed EGFR include the Src/STAT pathway, Ras/Raf/MEK/MAPK/ERK pathway, the Ras/PI3K/Akt/mTOR pathway and also the PKC pathway. Apart from the direct

upregulation of the overexpressed EGFR downstream effectors there are often coexistent abnormalities of EGFR and other molecules of the mTOR pathway. Interestingly, cells that highly overexpress the truncated, constitutively active mutant of the EGFR (EGFRvIII) preferentially use the PI3K pathway. This is a possible mechanistic basis for the success of PI3K and mTOR small molecule inhibitors incombination with EGFR kinase inhibitors in the treatment of EGFRvIII-expressing cells and xenografts. There is mounting evidence of enhanced antineoplastic activity and overcome of treatment resistance from the combination of EGFR inhibitors with inhibitors of mTOR kinase. Overexpression of the IRS-1 molecule has long been known to play role in the pathophysiology of hepatocellular carcinoma, causing arrest of apoptosis and induction of cell survival via upregulation of MAPK and PI3K molecules.

Although mTOR inhibitors have been extensively and successfully evaluated in hematological malignancies and transplant rejection treatment their role in non-hematological solid cancers is increasing. The main mTOR inhibitor, rapamycin (or sirolimus) target specifically the FKBP12-rapamycin-binding domain of the mTOR1 complex. There is some evidence that prolonged treatment with rapamycin may have an effect on mTORC2 and Akt, as well. As expected, due to their position only upstream to mTOR genetic and signaling abnormalities are subject to control by the rapamycin and its analogs whereas downstream molecules will remain unaffected by treatment with mTOR inhibitors.

DNA repair mechanisms are important in the resistance to cisplatin. The destruction of cells by cisplatin requires the binding of the drug to DNA and the creation of platinum-DNA adducts. Some of these adducts establish covalent cross-linking

between DNA strands, thereby inhibiting DNA replication. Nucleotide excision repair has a central role in DNA repair and is associated with resistance to platinium-based chemotherapy. The excision repair cross-complementation group 1 (ERCC1) enzyme plays a rate-limiting role in the nucleotide excision repair (NER) pathway that recognizes and removes cisplatin-induced DNA adducts. ERCC1 is also important in the repair of interstrand cross-links in DNA and in recombination processes. In vitro studies have linked platinum resistance to the expression of ERCC1 messenger RNA (mRNA) in cell lines involved in ovarian, cervical, testicular, bladder, and non-small-cell lung cancers. The relation between the expression of ERCC1 mRNA and resistance to platinium compounds has been corroborated by small, retrospective clinical studies in patients with advanced gastric, ovarian, colorectal, esophageal, or non-small-cell lung cancer. [14]

Paolo Ceppi, BSc et al demonstrated that among patients with LD SCLC those with low ERCC1 mRNA levels had significant longer survival than those with high ERCC1 expression (p=0,012) and may support the role of ERCC1 as potential predictive factor of survival in SCLC patients with LD. [12]. Although in this study no significance was found.

Brigit guldhammer Skov et al concluded ERCC1 expression in SCLC treated with platinum-based chemotherapy has no impact on survival. High expression of ERCC1 in TC might represent a clue to the failure of platinum-based therapy in these patients. [53]

In this study patients with negative score for ERCC1 had significantly better response to first line chemotherapy, log-rank p = 0,001. High proliferation rate is associated with tumor aggressiveness in many tumors, including NE. This might be the

result from genomic instability, which is the feature of tumors with low ERCC1 expression.

One possible mechanism by which SCLC cells can escape the effects of cytotoxic drugs was discovered in experiments elucidating SCLC responses to cisplatin, a DNA-damaging agent. Surprisingly, treatment with cisplatin up-regulated Akt activation and contributed to the expression of pro-survival proteins in SCLC cells. Additionally, it has been found that a rapamycin derivative (CCI-779) sensitized 2cisplatin-resistant SCLC cells to the effects of the cytotoxic drug. Unfortunately CCI-779 did not improve patient survival, when administrated as a single agent in a Phase II clinical trial for SCLC patients after chemotherapy induction. Collectively, these data demonstrate a significant role for the PI3K/Akt/mTOR pathway in the biology of SCLC. Moreover, the PI3K pathway was also shown to be activated through integrins in response to adhesion on extracellular matrix (ECM) molecules and this resulted in resistance of SCLC cells to various therapies and protection from apoptosis. Patients expressing positive mTOR staining had significant better survival when received the first-line chemotherapy. (log-rank p = 0,020) [54].

The exact mechanism of etoposide's antineoplastic effect is unknown. Etoposide is a topoisomerase II inhibitor. It seems to act at the premitotic stage of cell division to inhibit DNA synthesis; it is cell cycle–dependent and phase-specific, with maximum effect on the S and G_2 phases of cell division. Metabolism studies of the antitumor drug etoposide show the formation of metabolites in the lactone ring, which are probably not important for the drug's mechanism of action, and oxidative transformations in the dimethoxyphenol ring (E ring), which lead to products that can cause DNA damage and

may play a role in the drug's mechanism of action. The cytotoxicity of etoposide is caused by the induction of DNA damage. The occurrence of the DNA lesions can be explained by the capacity of the drug to interfere with the scission-reunion reaction of mammalian topoisomerase II by stabilizing a cleavable complex. [15].

Conclusions

This data indicates the possible prognostic value for mTOR in patients diagnosed with SCLC. ERCC1 lower expression and mTOR overexpression were correlated to longer survival in patients treated with etoposide+carboplatin. This piece of work reveals the importance to adopt individual based therapy that improves effective management of this devastating disease.

References

1. Jemal A, Murray T, Ward E, et al. (2005) Cancer Statistics. CA Cancer J Clin 55:10-30. [Erratum, CA Cancer J Clin 2005; 55:259].

2. Jackman DM, Johnson BE. Lancet 2005; 366(9494):1385-96.

3. Govindan R, Page N, Morgensztem D, et al. J Clin Oncol 2006; 24(28):4539-4544.

4. Schiller J, et al. (2001) Topotecan versus observation after cisplatin plus etoposide in extensive-stage small cell lung cancer: E7593-a Phase III trial of the Eastern Cooperative Oncology Group. J Clin Oncol 19:2114-2122.

5. Hanna NH, et al. (2002) Maintenance daily oral etoposide versus no further therapy following induction chemotherapy with etoposide plus ifosfamide plus cisplatin in extensive small-cell lung cancer: a Hoosier Oncology Group randomized study. Ann Oncol 13:95-102.

6. Steward WP, et al. (1998) Effects of granulocyte colony-stimulating factor and dose-intensification of V-ICE chemotherapy in small-cell lung cancer: a prospective randomized study of 300 patients. J Clin Oncol 16:642-650.

7. National Cancer Institute, U.S. National Institutes of Health website on small-cell lung cancer for health professionals. Available at: http://www.cancer.gov/cancertopics/pdq/treatment/small-cell-lung/healthprofessional/page2 Accessed on April 8, 2009.

8. Johnson BE, et al. (1996) Patients with limited-stage small-cell lung cancer treated with concurrent twice-daily chest radiotherapy and etoposide/cisplatin followed by cyclophosphamide, doxorubicin and vincristine 14:806-813.

9. Murray N, et al. (1993) Importance of timing for thoracic irradiation in the combined modality treatment of limited-stage small-cell lung cancer: The National Cancer Institute of Canada Clinical Trials Groups. J Clin Oncol 11:336-344.

10. Turrisi AT 3rd et al. (1999) Twice-daily compared with once-daily thoracic radiotherapy in limited small-cell lung cancer treated concurrently with cisplatin and etoposide. N Engl J Med 340:265-271.

11. Foster R. N., et al (2009) Prognostic factors differ by tumor stage for small cell lung cancer. Cancer 115(12):2721-2731

12. Ceppi P, et al. (2008) Excision Repair Cross Complementing-1 and Topoisomerase IIα Gene Expression in Small-Cell Lung Cancer Patients Treated with Platinum and Etoposide: a retrospective study. Journal of Thoracic Oncology 3(6):583-589.

13. Guldhammer B, et al. (2010) ERCC1 and Ki67 in Small Cell Lung Carcinoma and Other Neuroendocrine Tumors of the Lung: Distribution and Impact on Survival. Journal of Thoracic Oncology 5(4):453-459.

14. Olaussen K, et al. (2006) DNA Repair by ERCC1 in Non-Small-Cell Lung Cancer and Cisplatin-Based Adjuvant Chemotherapy. The New England Journal of Medicine No 10 Vol 355: 983-991.

15. You B, et al. (2008) Etoposide pharmacokinetics and survival in patients with small cell lung cancer: a multicentre study. Lung Cancer 62:261-272

16. Simon G, Ismail-Khan R, Bepler G (2007) Nuclear excision repair-based personalized therapy for non-small cell lung cancer: from hypothesis to reality. The International Journal of Biochemistry & Cell Biology 39:1318-1328.

18. Shaw RJ, Cantley LC (2006) Nature 441(7092):424-430

19. Bader AG, et al. (2005) Nat Rev Câncer 5(12):921-929.

20. Hennessy BT, et al. (2005) Nat Rev Drug Discov 4(12):988-1004.

21. Scott PH, et al. (1998) Proc Natl Acad Sci USA 95(13):7772-7777.

22. Nave BT, et al. (1999) Biochem J 344(Pt 2):427-431.

23. Ballif BA, et al. (2005) Proc Natl Acad Sci USA 102(3):667-672.

24. Ma L, et al. (2005) Cell 121(2):179-193.

25. Roux PP, et al. (2004) Proc Natl Acad Sci USA 101(37):13489-13494

26. Johannessen CM, et al. (2005) Proc Natl Acad Sci USA 102(24):8573-8578.

27. Faivre S, et al. (2006) Nat Rev Drug Discov 5(8):671-688.

28. Vignot S, et al. (2005) Ann Oncol 16(4):525-537.

29. Krystal GW, et al. (2002) Mol Cancer Ther 1(11):913-922.

30. Massion PP, et al. (2004) Am J Respir Crit Care Med 170(10):1088-1094.

31. Blackhall FH, et al. (2003) Clin Cancer Res 9(6):2241-2247.

32. Tsurutani J, et al. (2005) Cancer Res 65(18):8423-8432.

33. Vezina C, et al. (1975) Rapamycin (AY-22, 989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot (Tokyo) 28:721-726.

34. Brown EJ, et al. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature 369:756-758.

35. Donnelly JG, Soldin SJ (1994) Partial characterization of a 52 kDa CsA/FK506/ rapamycin binding protein. Clin Biochem 27:367-372.

36. Wiederrecht GJ, et al. (1995) Mechanism of action of rapamycin; new insights into the regulation of G1-phase progression in eukaryotic cells. J Biol Chem 270:815-822.

37. Vanhaesebroeck B, Waterfield MD (1999) Signaling by distinct classes of phosphoinositide 3-kinases. Exp Cell Res 253:239-254.

38. Jimenez C, et al. (1998) Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. EMBO J 17:743-753.

39. Shaw RJ, Cantley LC, Ras P (2006) PI(3)K and mTOR signalling controls tumour cell growth. Nature 441:424-430.

40. Festuccia C, et al. (2005) Molecular aspects of gefitinib antiproliferative and pró-apoptotic effects in PTEN-positive and PTEN-negative prostate cancer cell lines. Endocr Relat Cancer 12:983-998.

41. She QB, et al. (2003) Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3'-kinase/Akt pathway signaling. Clin Cancer Res 9:4340-4346.

42. Liaw D, et al. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 16:64-67.

43. Murata J, et al. (1999) Dysplastic gangliocytoma (Lhermitte-Duclos disease) associated with Cowden disease: report of a case and review of the literature for the genetic relationship between the two diseases. J Neurooncol 41:129-136.

44. Chiariello E, et al. (1998) PTEN/MMAC1 mutations in primary glioblastomas and short-term cultures of malignant gliomas. Oncogene 16:541-545.

45. Kohno T, et al. (1998) Inactivation of the PTEN/MMAC1/TEP1 gene in human lung cancers. Genes Chromossomes Cancer 22:152-156.

46. Rhey E, et al. (1997) Mutation analysis of the putative tumor supressor gene PTEN/MMAC1 in primary breast carcinomas. Cancer Res 57:3657-3659.

47. Strimpakos A, et al. (2009) The role of mTOR in the management of solid tumors: An overview. Câncer Treatment Reviews 35:148-159.

48. Dowling R, et al. (2010) Dissecting the role of mTOR: Lessons from mTOR inhibitors. Biochimica et Biophysica Acta 1804:433-439.

49. Carvalho L, et al. (2009) Revista Portuguesa de Pneumologia No 4 Vol XV:683-696

50. Sandler A. (2003) Chemotherapy for small cell lung cancer. Semin Oncol. 30:9-25.

51. Marinis F, et al. (2005) A Multicenter, Randomized, Phase II Study of Cisplatin, Etoposide and Gemcitabine or Cisplatin plus Gemcitabine as First-Line Treatment in Patients with Poor-Prognosis Small Cell Lung Carcinoma. Cancer 103(4):772-779.

52. Wolfgang M. et al (2006) Predictive and prognostic factors in Small Cell Lung Carcinoma (SCLC) – Analysis from routine clinical practice. Anticancer research 26: 4825-4832.

53. Skov, B et al (2010) ERCC1 and Ki67 in Small Cell Lung Carcinoma and other neuroendocrine tumors of the lung. Journal of Thoracic Oncology 5: 453-459

54. Marinov et al (2007) Targeting mTOR signaling in lung cancer. Critical reviews in Oncology/hematology 63: 172-182.

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