

Identification of a novel subpopulation of Caspase-4 positive Non-Small Cell Lung Cancer patients.

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Research

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Abstract

Background. Therapy/prognosis of Non-Small Cell Lung Cancer (NSCLC) patients are strongly related to gene alteration/s or protein expression. However, more than 50% of NSCLC patients are negative to key drugable biomarkers.

Methods: We used human samples of NSCLC and mouse models of lung adenocarcinoma.

Results. We showed that caspase-4 was highly present in the tumor mass compared to non-cancerous human tissues. Interestingly, the orthologue murine caspase-11 promoted lung carcinogenesis in mice. Carcinogen-exposed caspase-11 knockout mice had lower tumor lesions than wild type mice, due to the relevance of caspase-11 in the structural lung cell as demonstrated by bone marrow transplantation and adoptive transfer experiments. Similarly to what observed in mice, caspase-4 was correlated to the stage of lung cancer in humans in that it induced cell proliferation in a K-Ras, c-MyC and IL-1 α dependent manner. Caspase-4 positive adenocarcinoma (79.3%) and squamous carcinoma (88.2%) patients had lower median survival than patients who had lower levels of caspase-4. Moreover, PD-L1 expression and gene mutation (i.e. EGFR) were not correlated to caspase-4 expression. Instead, NSCLC patients who had K-Ras or c-MyC gene alteration were positively correlated to higher levels of caspase-4 and lower survival rate.

Conclusions. We identified a subgroup of NSCLC patients as caspase-4 positive among which double and triple positive caspase-4, K-Ras and/or c-MyC patients which prognosis was poor. Because K-Ras and c-MyC are still undruggable, the identification of caspase-4 as a novel oncoprotein could introduce novelty in the clinical yet unmet needs for NSCLC patients.

Background

Lung cancer is one of the leading causes of cancer-related death worldwide (1, 2). Epidemiological studies report that lung chronic inflammation initiate/promote the development of lung cancer, possibly in conjunction with tobacco use and/or other environmental pollutants (i.e. asbestos, silica, diesel exhaust). Epithelial cells, alveolar macrophages (MΦ) and resident dendritic cells (DCs) are the first line of defense for the respiratory tract. Their prolonged contact with insulting exogenous molecules can initiate and sustain inflammatory responses which signature could be IL-1 β dependent (3, 4), leading to chronic inflammation (3). In support, elevated serum levels of C-reactive protein (CRP) and high erythrocyte sedimentation rate (ESR) are both associated to lifestyle (i.e. smoking, air pollutant exposure) and are related to increased risk of lung cancer (5). Concomitantly, high levels of the pro-inflammatory cytokines, such as IL-1 β and IL-18, are detected in the plasma and tissue of lung cancer patients (6), identified as bad prognostic biomarkers for cancer patients (7). IL-1-like cytokines (i.e. IL-1 α , IL-1 β , IL-18 and IL-33) are identified as 'alarmins'. Their expression is tightly regulated by multiprotein complexes referred to as 'inflammasomes', which activation promotes caspase-1 cleavage into its active form with the ensuing activation of IL-1 β and IL-18 (8). Alternatively, non-canonical inflammasome engages

caspase-11 (also known as caspase-4 in humans) which can induce the release of alarmins such as IL-1 α , IL-1 β , IL-18 and HMGB1 (8). Human caspase-4, as well as the analogue murine caspase-11, was described as a pro-inflammatory caspase that can serve as host defense via the induction of pyroptosis to eliminate intracellular pathogens, , and via the release of pro-inflammatory IL-1-like cytokine (i.e. IL-1 α and IL-1 β , IL-18) in a canonical inflammasome pathway. Nevertheless, in this latter case it was demonstrated that caspase-11 unlikely processes IL-1 β and IL-18 in a direct manner (9), rather, it can promote the downstream caspase-1 activation via NLRP3 (9). On the other hand, IL-1 α release can be directly related to caspase-11 (10).

In our previous murine study, we reported that tumor-associated macrophages (TAMs) populated lung tumor lesions exerting a pro-tumor activity in a caspase-11/caspase-1-dependent manner, implying that the activation of the inflammasome in TAMs was pro-tumorigenic (11). Moreover, we found that NSCLC patients had higher circulating levels of caspase-4 than healthy subjects (12). In this study we demonstrated that caspase-4 was highly present in the tumor mass compared to non-cancerous tissues of NSCLC patients and was responsible for cell proliferation, suggesting it as a novel oncoprotein that collaborates with c-MyC and K-Ras to promote lung cancer, affecting patients' survival rate.

Materials And Methods

Human samples. Samples in this study were obtained by patients diagnosed of operable NSCLC (stage IA-IB, n=79; Stage IIA-IIB, n=34; Stage IIIA-IV, n=12), and underwent surgical resection at Ospedale dei Colli, AORN, Monaldi, Naples, Italy, during the period 2014-2017. Clinical data were obtained from questionnaires and histology reports from the Pathological Anatomy Unit of the hospital. The project was approved by the institutional review board and by the Ethical Committee (approval number for lung cancer patients 1254/2014). Samples from lung cancer patients were collected after oral and written information provided by the MDs, and after the signature of a consent form before entering the project. Samples were collected and used within 24 hours. Lung cancer patients were 60 \pm 10 (mean \pm S.E.M.) years of age. Biochemical analyses on PD-L1 and genetic mutations (i.e. EGFR mutations, ALK, ROS1 and MET genetic alterations) were performed by double blinded operator at Ospedale dei Colli, AORN, Monaldi, Naples, Italy. Survival data was analysed according to the period 2014-2017 and was related to the values of tissue caspase-4 after surgical resection. Human samples were collected and processed within 24 hours surgical resection.

Mice. Female specific pathogen-free C57BL/6 mice, B6N.129S2-Casp1<tm1Flv>/J, CASP1/11 knockout (ko), C3H/HeJ (6-8 weeks of age) (Jackson Laboratories, USA, and Charles River Laboratories, Lecco, Italy) were fed a standard chow diet and housed under specific pathogen-free conditions at the University of Salerno, Department of Pharmacy. CASP11 ko were kindly provided by Dr. Vishva Dixit from Genentech USA. Samples from transgenic mice K-Ras^{LA1} or K-Ras^{LA1}/p53^{R172HΔ} were kindly provided by Dr. Quaglino, University of Turin, Italy. All animal experiments were performed under protocols that followed the Italian and European Community Council for Animal Care (2010/63/EU). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of

the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Salerno and by National Institutes of Health with the approval number 13786/2014.

Experimental Protocol.

Mouse model of lung carcinogenesis. Mice were intratracheally (i.t.) instilled with N-methyl-N-nitroso-urea (NMU) at the dose of 50 μ g/mouse at week 1 (day 0), week 8 (day 56), week 12 (day 84), and the dose of 10 μ g/mouse was instilled at week 1 (day 7), week 2 (day 14), week 9 (day 63), week 10 (day 70), week 13 (day 91) week 14 (day 98), according to Figure 4A. Lungs were isolated and digested with 1U/mL collagenase (Sigma Aldrich, Milan, Italy). Cell suspensions were passed through 70 μ m cell strainers, and red blood cells were lysed. Cell suspensions were used for flow cytometric analyses of different cell subtypes. Broncho-alveolar lavage fluid (BAL) was collected using 0.5 ml of PBS containing 0.5mM EDTA and cell counts performed. In addition, lungs were homogenized and cytokines measured.

Bone-marrow (BM) transplant. Bone-marrow (BM) transplant experiments were performed using wild type (wt, C57Bl/6 mice) and Caspase-11 ko mice. BM-derived cells were isolated from euthanized donor mice. Recipient 6-8 weeks old mice were irradiated with one dose of 10Grad to deplete endogenous BM stem cells and most of the BM-derived cells, before the transplantation of 1x10⁶ donor BM cells, injected into the tail vein of recipient irradiated mice.

Animals were divided in four groups:

1. wt into wt: donor wt cells into recipient wt mice;
2. ko into ko: donor Caspase-11 ko cells into recipient Caspase-11 ko mice;
3. wt into ko: donor wt cells into recipient Caspase-11 ko mice;
4. ko into wt: donor Caspase-11 ko cells into recipient wt mice.

The degree of chimerism was assessed by FACS analysis of CD45.1⁺ blood leucocytes 7-8 weeks after BM transplant. NMU or vehicle were instilled starting at 8 weeks post BM transplant and chimera mice were sacrificed 28 days after the first NMU exposure.

Flow Cytometry Analysis. Cell suspensions obtained by collagenase digested lungs were analysed to evaluate the infiltration and the nature of *immune cells* recruited to the lung of mice. *Cell suspensions were labelled with specific antibodies (CD11b, Gr-1, CD4, CD25, FoxP3).*

Western blotting analysis. Lung homogenates were used to examine the expression of caspase-4, in humans, (ImmunePharma srl, Italy) or caspase-11, in mice (Santa Cruz Technologies, CA, USA), kRas (AbCam, Cambridge, UK) by means of SDS- or Native-PAGE. Data were analysed by means of ImageJ (NIH, USA).

ELISAs. The presence of tissue caspase-4 was detected by an ELISA kit patented by ImmunePharma s.r.l. (RM2014A000080 and PCT/IB2015/051262) (Department of Pharmacy, University of Salerno, Italy). Custom antibodies were projected by ImmunePharma s.r.l., and they are not currently commercially available. The diagnostic performance of the custom antibodies has been previously described (12). Tissue caspase-4 expression was compared to caspase-5 by means of ELISA. IL-1 α and IL-1 β were measured in BAL or lung homogenates as specified in the text, using commercially available ELISA kits (eBioscience, CA, USA). In the first case cytokine levels were expressed as pg/ml in BAL samples, whereas in lung homogenates as pg/mg protein.

Immunohistochemistry. Human samples of lung tumor were embedded in paraffin to perform tissue microarray (TMA). Patient's characteristics are reported in Table I. NSCLC patients were considered as Caspase-4 positive (+) (Table I) according to a histological score that was calculated by a blinded and certified pathologist at the National Cancer Institute "Fondazione G. Pascale" (Naples, Italy). In particular, positive score was defined as positive area to caspase-4 detection that resulted $\geq 25\%$ compared to negative area ($\leq 25\%$). Human samples analysed by TMA were different from those for whom survival rate is described. A custom antibody against caspase-4, provided by ImmunePharma srl, Italy, was used to perform immunohistochemistry analyses. The diammino-benzidinic acid (DAB) system was used to detect complexes. Mouse IgG was used as an isotype control (ImmunePharma srl, Italy).

Table 1. General characteristics of the study participants and difference of intention to stay across different categories of participants' characteristics

		n	percentage	mean	standard deviation	P-value
Retention	Stay	636	87.8			
	Leave	88	12.2			
Sex	Male	31	4.4	29	93.5	0.307
	Female	681	95.6	595	87.4	
Age	Below 29	414	57.9	359	86.7	0.099
	30~39	221	30.9	191	86.4	
	40~49	65	9.1	63	96.9	
	Over 50	15	2.1	14	93.3	
Education level	Junior high school	4	0.6	4	100	0.327
	Junior college	295	41.2	258	87.5	
	Two-year college	44	6.1	43	97.7	
	Two-year technical college	163	22.8	139	85.3	
	University	191	26.7	168	88.0	
	Graduated school	19	2.7	16	84.2	
Religion	None	375	52.5	328	87.5	0.408
	Buddhism	201	28.2	178	88.6	
	Taoism	94	13.2	83	88.3	
	Christianity	21	2.9	20	95.2	
	Catholic	9	1.3	6	66.7	
	Other	14	2.0	12	85.7	
Seniority of nursing work	Within 1 year	109	15.2	101	92.7	0.003**
	1~3 year	169	23.6	152	89.9	
	3~5 year	118	16.5	98	83.1	
	5~10 year	125	17.5	99	79.2	
	Over 10 year	195	27.2	178	91.3	
Seniority of hospital work	Within 1 year	135	18.9	124	91.9	0.067
	1~3 year	194	27.1	172	88.7	
	3~5 year	133	18.6	110	82.7	
	5~10 year	141	19.7	119	84.4	
	Over 10 year	112	15.7	103	92.0	
Time of received medical and human-related education and training in the past six months	None	90	12.6	76	84.4	0.426
	1~5 hours	499	69.7	437	87.6	
	6~10 hours	102	14.2	94	92.2	
	Over 10 hours	25	3.5	22	88.0	

Note: *p<0.05, **p<0.01, ***p<0.001

Mice left lung lobes were fixed in OCT medium (Pella Inc., Milan, Italy) and 7 µm cryosections were cut. H&E staining was performed and used to measure the tumour burden. Tumor lesions were analysed by means of Image J (NIH, USA) and expressed as Tumor lesions=ratio tumor area/total lung area, as already reported (13). Lung tumor area and the hyperplastic cells were counted by using serial lung cryosections in a blinded fashion.

Reverse Transcriptase-Polymerase Chain Reaction and Real-Time Polymerase Chain Reaction.

Total RNA was isolated from lung tissue samples by using the RNeasy Mini extraction kit according to the manufacturer's instructions (Qiagen, United Kingdom). Reverse Transcription was performed by using first-strand cDNA synthesis kit (Qiagen, United Kingdom) followed by PCR, as already reported (37). Thermal cycling conditions for caspase-4 were 5 min at 95°C, followed by 45 cycles of 45 s at 94°C, 30 s at 60°C, 30 s at 72°C.

Thermal cycling conditions for c-MyC were 5 min at 95°C, followed by 45 cycles of 45 s at 94°C, 30 s at 66°C, 30 s at 72°C.

Primer pairs were as follow:

-Caspase 4 (NM_001225.3): Forward 5'-TTTCTGCTCTTCAACGCCAC-3'; Reverse 5'-AGTCGTTCTATGGTGGGCAT-3';

-c-MyC (REF NM_002467): Forward 5'-AAAGGCCCCCAAGGTAGTTA-3'; Reverse 5'-GCACAAGAGTTCCGTAGCTG-3'

- β -actin: Forward 5'-AGAGCTACGAGCTGCCTGAC-3'; Reverse 5'-AGCACTGTGTTGGCGTACAG-3'

RT-PCR for k-Ras was performed according to the MGBE probe following manufacturer's instructions (PrimeTime Gene expression Mastermix kit, IDT, USA) were 3 min at 95°C, followed by 50 cycles of 30 s at 95°C, 30 s at 54°C, 30 s at 80°C. Primers were as follows:

k-RAS^{G12C}: Forward 5'-AATATAAACCTTGTGGTAGTTGGAGCCT-3'

k-RAS^{G12D}: Forward 5'-AAACTTGTGGTAGTTGGAGCGGA-3'

k-RAS^{G12V}: Forward 5'-AAACTTGTGGTAGTTGGAGCAGT-3'

k-RAS: Reverse 5'-CATATTCGTCCACAAAATGATTCTG-3'

Probe: 5'-/56-FAM/CTGTATCGTCAAGGCCT/3MGBEc/-3'

Lung cell transfection. A549 cells, adenocarcinomic human alveolar basal epithelial cells, were purchased from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS, L-Glutamine (2mM), penicillin (100U/ml) and streptomycin (100 μ g/ml) (Sigma-Aldrich, Milan Italy) in an atmosphere of 5% CO₂ at 37°C. Cell transfection was performed following manufacturer's instructions (Transit kit, Mirus Bio Inc., USA). Caspase-4 sequence (NM_001225.3) was encoded into pcDNA3.1+C-6His and used for cell transfection at the concentration of 50ng/ml (Genscript Inc., Netherlands). In particular, four different pcDNA plasmids were used according to the caspase-4 mRNA sequence encoded: 1. pcDNA-1 (PC-1): sequence from nucleotide (nt) 74-1205; 2. pcDNA-2 (PC-2): sequence from nt 74-810; 3. pcDNA-3 (PC-3): sequence from nt 348-1205; 4. pcDNA-4 (PC-4): sequence from nt 423-886. Empty vector was used as negative control.

Cell proliferation assay. Transfected and non-transfected A549 cells were previously marked by using carboxyfluorescein diacetate succinimidyl ester (CFSE; 5µM; Molecular Probes, Invitrogen) to perform proliferation assay. CFSE flow cytometry data was analyzed by means of ModFit4.0 software (BD Pharmingen). In some experiments, non-transfected A549 cells were treated with human recombinant (ImmunePharma srl., Italy) of the large subunit of caspase-4 (100ng/ml) and co-cultured with peripheral blood mononuclear cells (PBMCs) obtained by NSCLC patients (ratio 1:5). PBMCs were isolated by means of Ficoll's protocol as previously reported (Molino et al., 2019).

In another type of experiments, transfected A549 cells were treated with specific pharmacological inhibitors, such as anti-EGFR (10µg/ml, AbCam, UK, FTI-276 (5µg/ml, k-Ras inhibitor, Sigma Aldrich, Rome, Italy), SAHA (5µg/ml, histone deacetylase, HDAC, inhibitor, Sigma Aldrich, Rome, Italy), 5-AZA (5µg/ml, DNA methylase inhibitor, Sigma Aldrich, Rome, Italy) and rapamycin (1µg/ml, mTOR inhibitor, Sigma Aldrich, Rome, Italy).

Statistical Analysis. Data are reported as median ± percentile range and represented as violin plots. Statistical differences were assessed with TWO-WAY or ONE-WAY Analysis of variance (ANOVA) followed by multiple comparison post-tests as appropriate. Percent survival was estimated by means of Kaplan-Meier method and compared with a non-parametric log-rank test. Percent survival was calculated from the time of surgical resection. The survival rate was calculated for 73 patients which tissue-derived biological samples could be tested by means of the ELISA kit. p values less than 0.05 were considered significant.

Results

1. Caspase-4 is involved in Non-Small Cell Lung Cancer.

Recently, we demonstrated that NSCLC patients had higher circulating levels of caspase-4 than healthy subjects (12). In this study we found that NSCLC tissues had a positive histological score of caspase-4 which was higher in the tumor masses than in non-cancerous tissues (Figure 1A). In particular, as already observed by using a different technical approach (12), more than 70% of patients presented tissue positivity to caspase-4 (Table I). Similarly, transcriptional levels of CASP4 mRNA were higher in the tumor mass than in healthy (non-cancerous) tissues of NSCLC patients (Figure 1B). To note, peritumoral areas had intermediate levels of caspase-4 mRNA compared to healthy (non-cancerous) and tumoral areas (Figure 1B). These data were confirmed by means of western blotting (Figure 1C and D), which showed that lung cancer tissues were characterized by the presence of the cleaved form of caspase-4 (25-30 kDa) than healthy tissues. Based on these data, we hypothesized that the presence of caspase-4 could be related to the TNM stage of the pathology. Interestingly, the cleaved form of caspase-4, herein identified as tumor-associated caspase-4, statistically increased from stage I up to stage III (Figure 1E, stage I to III). To correlate the malignancy stage to the levels of tissue-associated caspase-4, we chose a cut-off value of the protein, as already reported (12) and correlated it to the survival rate. The survival rate of NSCLC patients at stage I who presented higher levels (>377 pg/ml) of tissue-associated caspase-4

was lower (median=0.967 years) than patients at stage I who presented lower (<377 pg/ml) levels (median=3.02 years) (Figure 1F). Similarly, NSCLC patients at stage II had lower median survival rate when tumor-associated caspase-4 levels were higher than 377 pg/ml (Figure 1G). However, in this last case we were not able to reach statistical significance due to the low number of patients. Moreover, stage III NSCLC patients had worse survival rate (median= 0.65 years) when tumor-associated caspase-4 was >377 pg/ml compared to patients who had lower levels (median= not revealed; n=2) (Figure 1H). Again, it has to be pointed out that the number of patients at stage III were low.

Caspase-4 in humans and caspase-11 in mice have been widely associated to the non-canonical pathway of the inflammasome (8; 9). Therefore, we went on by analyzing the levels of tissue IL-1 β and IL-1 α , two cytokines derived by the inflammasome activation (8, 11, 14). We found that, despite what reported in literature (15, 16), NSCLC patients did not have differential levels of tissue IL-1 β (Figure 1I). Instead, we found that IL-1 α was significantly increased in the tumor tissues than non-cancerous tissues (Figure 1J). Because we already demonstrated a correlation between IL-1 α and caspase-4 (4, 17), we found that patients who presented levels of IL-1 α >1.39pg/mg protein (cut-off value chosen according to ROC analysis) in the tissue (black line, Figure 1K) had similar survival rate as patients who had IL-1 α >1.39pg/mg protein and caspase-4>377 pg/ml (Figure 1K, red line), compared to patients who had lower levels of both caspase-4 and IL-1 α (Figure 1K, green and blue lines).

Taken together these data suggest that tumor tissues are characterized by high levels of caspase-4 and IL-1 α which are associated to lower survival rate of NSCLC patients.

2. Higher levels of tumor-associated caspase-4 are present in the lung of NSCLC patients with poor survival rate.

To better understand the role of caspase-4 in lung cancer, we took advantage of a public database (www.cbioportal.org) where we analyzed what already reported in the literature regarding this enzyme. We found that 2% of patients in the public database presented a genomic alteration of CASP4 gene, intended as mutation or amplification or higher gene copy number. In this pattern we analyzed positive correlation between CASP4 and other genes according to Spearman correlation coefficient >0.25 taking into consideration both adenocarcinoma and squamous carcinoma patients reported in the database. In adenocarcinoma patients, CASP4 positively correlated to 9 genes involved in gene expression (i.e. C-MYCT1, SRA1, GTF2B, EEF1A1, SP110, CNOT8), 186 genes involved in inflammation (i.e. CASP1-5, CARD16, CD63, HLA, IRF1, TLRs), 106 genes involved in cell proliferation (i.e. C-MYCT1, SRA1, RHOA, CDK7, RAB32, CD74, RRAS) and 17 genes involved in cell death (i.e. CASP5, ANXA5, BAK1, RIPK2, MLKL) (Figure 2A). Similarly, in squamous carcinoma CASP4 positively correlated to 47 genes involved in gene expression (i.e. CREM, BATF, BZW1, HDAC9, STAT1-4), 303 genes involved in inflammation (i.e. AIM2, CASP5, CARD6-16-17-19, CCLs, ICOS, TLRs), 266 genes involved in cell proliferation (i.e. FAM107B, FGF7, IRF1-8-9, RASGRP3) and 63 genes involved in cell death (i.e. CASP8, ANXA3-5, FAS, GZMs, RIPK3) (Figure 2B). Among these, we found that caspase-5 and K-Ras correlated to CASP4 in both histotypes (Figure 2A and B, common areas in the Venn diagram).

In order to understand the impact of caspase-5 in NSCLC, we used a caspase-5 antibody to test via an ELISA assay the presence of this enzyme in tumor tissues compared to caspase-4, which was detected by means of ELISA by using a specific antibody, different from what is commercially available, actually under patent (RM2014A000080 and PCT/IB2015/051262). ROC analyses showed that the detection of caspase-4 in lung tumor masses was an excellent diagnostic tool for both adenocarcinoma (Figure 2C, red line) and squamous carcinoma (Figure 2D, red line) compared to caspase-5 (Figure 2C and 2D, green line). These data are in support to what already published (12), but further highlights that caspase-5, differently than caspase-4, is not associated to the cancerous pattern as it is also present in non-cancerous tissues. This effect was better described by the value of the AUC which was 0.5739 in adenocarcinoma (Figure 2C, green line) and 0.5385 in squamous carcinoma tissues (Figure 2D, green line). In support, adenocarcinoma patients who had tumor-associated levels of caspase-4 higher than 377 pg/ml (n=42/53, 79,3%) survived less (median=0.925 years) than patients with lower levels (n=11/53, 20,7%; median survival =3.03 years) (Figure 2E). Similar scenario was observed for squamous carcinoma patients. Higher tumor-associated caspase-4 (n=15, 88.2%) was associated to lower survival rate (median=0.86 years) than those who had lower levels (n=2, 11.8%; median= undefined) (Figure 2F). These data, despite the low number of patients in Figure 2F, most likely imply that tumor-associated caspase-4, but not caspase-5, is correlated to lung carcinogenesis.

3. Higher levels of tumor-associated caspase-4 are present in the lung of PD-L1 negative NSCLC patients.

NSCLC patients could be classified according to mutations and PD-L1 positivity (2). Therefore, we stratified NSCLC patients as PD-L1 positive vs PD-L1 negative according to the levels of tumor-associated caspase-4. We found that NSCLC patients who had higher levels of tumor-associated caspase-4 (>377 pg/ml, cut-off) but were negative for PD-L1 had lower survival rate (median survival=0.96 years; % of survival rate at 1 year≈30%) (Figure 3A, blue line) (Figure 3B, n=52/75, 69.3%) than patients (9.3%) who had higher levels of tumor-associated caspase-4 but PD-L1 positive (median survival=undefined; % survival rate at 1 year≈80%) (Figure 3A, black line; Figure 3B, n=7/75, 11.1%). However, patients with higher levels of tumor-associated caspase-4 but positive for PD-L1 (Figure 3A, black line) showed lower survival rate ($p=0.033$) than patients with low caspase-4 but positive for PD-L1 (Figure 3A, green line, n=1/75, 1.3%, median survival=undefined). Nevertheless, it has to be noted that there was a group of patients who were PD-L1 negative and presented lower tumor-associated caspase-4 (Figure 3A, red line; Figure 3B, n=15/75, 20%, % survival at 1 year=85%) whose median survival rate was of 2.98 years. These latter group survived more than patients with high levels of caspase-4 and PD-L1 negative (Figure 3A, blue line), survival rate that was still lower than patients who had PD-L1 positivity and high caspase-4 (Figure 3A, black line) and PD-L1 positivity and low caspase-4 (Figure 3A, green line). These data imply that a group of patients (Figure 3B, n=15/75, 20%), whose survival is still low, is independent from caspase-4 and PD-L1. Though, it is noteworthy that the survival of this group at 1 year is ≈80% (Figure 3A, red line) compared to the group with high caspase-4 and PD-L1 negative (Figure 3A, blue line), who instead, presented a survival rate at 1 year of ≈30%. Based on these analyses, among patients who presented with high levels of caspase-4 (Figure 3B, red bar), those who were negative for PD-L1 represented 88.1% (Figure 3C) compared to PD-L1 positive patients (Figure 3C, 11.9%), implying a further

stratification of NSCLC patients. Moreover, according to chi squared test, caspase-4 and PD-L1 were independent biomarkers as also observed in Figure 3B and 3C where caspase-4 positive and PD-L1 negative patients were the majority of patients in our database.

4. Higher levels of tumor-associated caspase-4 are present in the lung of K-Ras and c-MyC-mutated NSCLC patients.

Another important issue for NSCLC patients is about gene mutation/s (2). In this latter case, we stratified patients who presented EGFR mutation or MET, ROS1 or ALK translocation, herein defined as mutated (Mut^+), and compared them to the levels of tumor-associated caspase-4. We found that NSCLC patients who had high levels of tumor-associated caspase-4 ($>377 \text{ pg/ml}$) and did not have any gene mutation (Mut^-) (Figure 3D, blue line) had lower survival rate (median survival=0.95 years; % of survival rate at 1 year $\approx 35\%$) (Figure 3E, n=53/74, 71.6%) than patients who had higher levels of tumor-associated caspase-4 and were Mut^+ (survival at 1 year $\approx 80\%$) (Figure 3D, green line; Figure 3E, n=6/74, 8.1%). As observed before, there was a group of patients who were Mut^- and had lower tumor-associated caspase-4 (Figure 3D, red line; Figure 3E, n=14/74, 18.9%). This group of patients had a median survival rate of 2.98 years and however, had higher survival rate than patients with higher levels of caspase-4 and Mut^- (Figure 3D, blue line). In addition, the survival rate of patients who had Mut^+ and high caspase-4 (Figure 3D, green line) was still lower than Mut^+ and low caspase-4 (Figure 3D, black line), implying a group of patients (Figure 3E, n=14, 18.9%) where the survival is still low without any relationship with caspase-4. The survival rate of this group at 1 year was of $\approx 85\%$ compared to the group with high caspase-4 but Mut^- (Figure 3D, blue line), that instead presented survival rate at 1 year of $\approx 35\%$. Based on these analyses, among caspase-4 positive patients (Figure 3F, red bar) those who were Mut^- represented 89.8% (Figure 3F) compared to Mut^+ patients (Figure 3F, 10.2%). Moreover, according to chi squared test, caspase-4 and genetic alterations were independent biomarkers as also observed in Figure 3E and F where caspase-4 positive and Mut^- patients were the majority of patients in the database.

As shown in the Venn diagram (Figure 2A-B), caspase-4 was associated to genes involved in cell proliferation, such as K-Ras and c-MyC, well-known to be involved in lung carcinogenesis (2, 18, 19). Therefore, we analyzed both K-Ras mutations (G12C, G12D and G12V) and c-MyC expression related to caspase-4 levels in our experimental human samples. We found that NSCLC patients with high levels of tumor-associated caspase-4 and c-MyC positive (c-MyC $^+$) (Figure 3G, black line) had a median survival of 1 year, lower than patients who had lower levels of caspase-4 and c-MyC $^+$ (Figure 3G, green line; median survival=undefined). Patients who presented no positivity to both caspase-4 and c-MyC had longer survival than the other groups (Figure 3G, red line, median survival=3.028). Interestingly, patients with high levels of tumor-associated caspase-4 but who were c-MyC negative (c-MyC $^-$) still had lower survival rate (median survival=2.036 years) (Figure 3G, blue line), although it was higher than patients positive for both Caspase-4 and c-MyC (Figure 3G, black line). The survival rate at 1 year was of 50% for Caspase-4-c-MyC $^+$ patients, of $\approx 95\%$ for Caspase-4-c-MyC $^-$ and of $\approx 85\%$ for Caspase-4 negative c-MyC $^+$ and Caspase-4 positive c-MyC $^-$ patients (Figure 3G). Based on these analyses, patients who presented with

high levels of caspase-4 (Figure 3H, red bars) and c-MyC+ were 66.7% in our database (Figure 3I), implying that caspase-4 and c-MyC gene overexpression were strictly associated.

Similarly, we stratified patients according to the positivity to mutated K-Ras (G12C, G12D and G12V). We considered as positive those patients who presented at least one of the three mutations. Very interestingly, the survival rate of caspase-4⁺ and K-Ras⁺ patients was significantly reduced (0.97 years) (Figure 3J, black line) compared to patients who were caspase-4⁻ and K-Ras⁻ (Figure 3J, red line). Moreover, both groups of patients who were caspase-4⁺ and K-Ras⁻ or caspase-4⁻ and K-Ras⁺ had median survival rate of 2.07 and 3.028 years, respectively, further highlighting the relevance of caspase-4 (Figure 3J and 3K, green and blue line). To date, we found that the majority of patients were caspase-4⁺ and K-Ras⁺ (Figure 3L, 85.2%), compared to caspase-4⁺ but K-Ras⁻ patients (Figure 3L, 14.8%).

In addition, we stratified patients as caspase-4, c-MyC and K-Ras triple positive. Patients who were positive for the three targets had a survival rate of 0.98 years (Figure 3M, black line), similar to that observed for caspase-4 and K-Ras⁺ (Figure 3J, black line, median survival=0.97 years) and c-MyC⁺ (Figure 3G, black line, median survival=1 year) patients. This group of patients represented ≈74.1% (Figure 3N-O). Nevertheless, patients who were solely positive for caspase-4 still represented ≈7.4% of the study population (Figure 3N-O).

These data highlight that caspase-4 collaborates with c-MyC and K-Ras leading to a bad prognosis of NSCLC patients.

5. Caspase-11 facilitates lung tumor progression in mice.

Experimental biological samples showed that caspase-4 is related to poor survival of NSCLC patients. To better investigate the molecular mechanism, we went on by taking advantage of a mouse model of adenocarcinoma (13). C57Bl/6 mice (wild type) and Caspase-11 knockout (Caspase 11-ko) were i.t. injected with NMU at week 1-2-3, 8-9-10, 12-13-14 and sacrificed at week 16 (Figure 4A). Very importantly and in support to our previous human data, caspase-11 ko mice robustly developed lower lung tumor lesions than wild type mice (Figure 4B). Because LPS, a TLR4 ligand, was supposed to be able to induce caspase-11 activation via a second signal model (20), to rule out the role of TLR4, we used C3H mice who are defective in TLR4 signalling (21, 22). Surprisingly, C3H mice had similar lung tumor lesions as C57Bl/6 wild type mice (Figure 4C), implying that caspase-11-related pro-tumor activity is directly involved in lung tumor progression in mice. Therefore, because we previously demonstrated that the majority of NSCLC patients were caspase-4 and K-Ras⁺, we used lung tissues of K-Ras^{LA1} and K-Ras^{LA1}p53^{R172HB} mice who were previously described by Prof. Quaglino's laboratory (23) as a lung adenocarcinoma model that shows lung tumor lesions starting from 10 weeks of age. K-Ras^{LA1} mice, similar to C57Bl/6 mice injected with NMU, were characterized by cleaved caspase-11 (p25 fragment) (Figure 4D). Moreover, K-Ras^{LA1}p53^{R172HB} mice, who were characterized by mutated K-Ras and p53 and high lung tumor lesions (23), showed not only p25 but also p10 fragment (Figure 4D). Noteworthy, the precursor form of caspase-11 was higher expressed in these latter mice compared to NMU-injected

C57Bl/6, control (Ctr) mice (Figure 4D), implying that not only K-Ras mutation, but also p53 genetic alteration could be implied in caspase-11-related pro-tumor activity in mice.

Caspase-11 was described as involved in the non-canonical inflammasome pathway and associated to IL-1-like cytokine release (10, 11, 13, 17, 20). We found that IL-1 β was reduced in the BAL of caspase-11 ko mice at longer time point (16 weeks) (Figure 4E) compared to wild type mice. Instead, as also observed in human samples (Figure 1J), IL-1 α was significantly higher in the lung of wild type mice than caspase-11 ko mice (Figure 4F) at all-time points (30-56-116 days), implying a major role of IL-1 α . To better understand the relevance of IL-1 α vs IL-1 β in our experimental conditions, we injected mice with a monoclonal antibody against IL-1 α or IL-1 β . The neutralization of IL-1 α significantly reduced the number of hyperplastic cells in the lung of NMU-injected C57Bl/6 mice (Figure 4G, all-1 α) compared to the control group (CTR) and mice who were similarly treated with a neutralizing antibody against IL-1 β . The IgG isotype control group showed similar hyperplastic cell number (0.133±0.07) as CTR mice (0.139±0.06).

Tumor immunosuppression has been widely described as pivotal for cancer progression (11, 13, 24). Therefore, we moved on to analyze the immune microenvironment in the lung of NMU-treated tumour-bearing mice. The percentage of myeloid-derived suppressor cells (MDSCs; identified as CD11b $^+$ Gr-1 high) were significantly reduced in the lungs of NMU-treated caspase-11 ko mice compared with wild type (Figure 4H). In contrast, the percentage of T regulatory cells (Treg: identified as CD4 $^+$ CD25 $^+$ FoxP3 $^+$ cells) was not altered in caspase-11 ko mice compared to wild type (Figure 4I), implying that the adaptive immunity was not affected by the genetic absence of caspase-11. Therefore, we injected NMU-treated Nude mice with isolated CD4 $^+$ cells obtained from wild type or caspase-11 ko mice (Figure 4A). We found that NMU-treated Nude mice, although with a tendency to reduction, not statistically significant, had similar lung tumor lesions as wild type (C57Bl/6) mice (Figure 4J). Interestingly, neither the adoptive transfer of wild type CD4 $^+$ T cells nor the adoptive transfer of caspase-11 ko CD4 $^+$ T cells altered lung tumor lesions (Figure 4J), implying that caspase-11 has no relevance in the adaptive immunity. In contrast, bone marrow transplantation experiments showed that caspase-11 ko mice receiving wild type bone marrow-derived cells (Figure 4K) had the lowest number of lung tumor lesions compared to wild type mice receiving ko bone marrow-derived cells (Figure 4K; ko into wt vs wt) and wild type lung tumor-bearing mice (Figure 4K; wt into ko vs casp 11 ko). To note, NMU-treated caspase-11 ko mice receiving wild type bone marrow-derived cells had similar levels of tumor lesions as NMU-treated caspase-11 ko mice (Figure 4K, wt into ko vs casp 11 ko), implying that the structural caspase-11 is involved in lung cancer progression in mice. Nevertheless, NMU-treated wild type mice receiving ko bone marrow-derived cells (Figure 4K; ko into wt) still had lower lung tumor lesions compared to NMU-treated wt mice, although the tumor lesions was higher than those in caspase-11 ko mice receiving wild type bone marrow-derived cells (Figure 4K; ko into wt vs wt into ko). Taken altogether, these data suggest that caspase-11 is involved in lung tumor progression in mice influencing the innate immunity most likely via IL-1 α .

6. Large subunit of Caspase-4 facilitates tumor proliferation.

The above data imply that caspase-4 in the structural cell component of lung tissues can favor tumor formation and progression. Therefore, to go inside the molecular mechanism we transfected A549 cells, mimicking lung epithelial cells, with viral vectors to express the long structure of caspase-4 (mRNA: 74-1205 nucleotides, nt, CARD+LARGE+SMALL subunit, PC4-1), the structure of the protein with CARD+LARGE subunit (mRNA: 74-810 nt, PC4-2), the structure of the protein with LARGE+SMALL subunit (mRNA: 348-1205 nt, PC4-3) and the structure of the protein with the sole LARGE subunit (mRNA: 423-886 nt, PC4-4). Lung epithelial cells transfected with PC4-1, PC4-2 and PC4-3 showed higher proliferation rate than control and empty vector-transfected cells (Figure 5A). Because the three vectors had a common nucleotidic sequence which corresponded to the LARGE subunit and because both human and mouse samples showed p25 fragment of caspase-4 and caspase-11 in lung homogenates, we investigated the role of this subunit. Interestingly, we found that cells transfected with PC4-4, which solely contained nucleotides for the LARGE subunit, robustly proliferated compared to control and empty vector transfected cells, but also to PC4-1, PC4-2 and PC4-3 transfected cells (Figure 5A). Similarly, treatment of cells with the recombinant protein that mimicked the large subunit of caspase-4 increased cell proliferation compared to the control (Figure 5B; white violin plot). Moreover, the addition of NSCLC patient-derived PBMCs did not alter the proliferation of cells (Figure 5B, green bars), further highlighting what was previously observed in mice about the role of caspase-11 in lung structural cells.

In order to understand the molecular mechanism underlying caspase-4-induced cell proliferation, we treated cells with cetuximab, a monoclonal antibody against EGFR. Cell proliferation was not altered when cetuximab was added to caspase-4-treated cells (Figure 5C), implying that EGFR signaling was not involved. Similarly, the inhibition of histone deacetylase (HDAC), highly involved in lung cancer (27), by means of SAHA (Figure 5D) and of DNA methyltransferase by means of 5-AZA (Figure 5E) did not modify caspase-4-induced cell proliferation, implying that caspase-4 is unlikely to be involved in epigenetic modulation/s that lead to tumor cell proliferation (28). In sharp contrast, the inhibition of K-Ras by means of FTI significantly reduced caspase-4-induced cell proliferation (Figure 5F, red bars). In support, we found that caspase-4 and K-Ras co-immunoprecipitated in lung tumor tissues (Supplementary Figure 1). To rule out another proliferative pathway, we treated cells with rapamicin, mTOR inhibitor. Again, we did not find an alteration in caspase-4-induced cell proliferation (Figure 5G, blue bars). These data suggest that the large subunit of caspase-4 induces cell proliferation via K-Ras pathway. In support, we found that caspase-4⁺ and K-Ras mutated patients had lower survival rate than other NSCLC patients, supporting the biochemical analyses.

Discussion

In this study we found that caspase-4 is correlated to lung carcinogenesis and poor survival rate of NSCLC patients. Herein, caspase-4 could be identified as a novel oncoprotein since 79.3% and 88.2% of adenocarcinoma and squamous NSCLC patients, respectively, stained positive for the protein which pro-tumor activity was reflected in a concerted cooperation with mutated K-Ras and cMyC. Interestingly, a

subpopulation of NSCLC patients (20 out of 35=57.1%) were triple positive for caspase-4, mutated K-Ras and c-MyC and presented a survival rate of less than 1 year.

Caspase-4 in humans and the analogue murine caspase-11 have been widely described as inflammatory caspases involved in the non-canonical inflammasome pathway in that they are able to sense LPS and lead to the release of IL-1 β and IL-18 other than inducing pyroptotic cell death (8, 25), identified by the release of LDH from cells. One limitation of our study, though, is that the endogenous ligand for caspase-4 in lung cancer is still not identified; however, our data demonstrate that caspase-4/caspase-11 are involved in lung carcinogenesis in humans and mice, respectively. This is the first study, to our knowledge, to show the pro-tumorigenic role of caspase-11 in mice and caspase-4 in humans. Caspase-11 activation is regulated via the TLR4/IFN pathway upon TRIF-induced procaspase-11 processing (9). In this regard, we previously demonstrated that the administration of Poly I:C, a TLR3 ligand, that is solely regulated by TRIF and that leads to IFN type I release, reduced tumor burden in a syngenic lung cancer mouse model (26). It is noteworthy that Poly I:C-induced reduction of lung tumor in mice was strictly related to the activation of the innate immunity against the tumor. Instead, in this study, we found that caspase-11 is significantly relevant in the structural cell compartment where it is involved in lung carcinogenesis in mice. Indeed, bone marrow transplantation of wild type cells into NMU-treated caspase-11 ko mice, as well as in the case of NMU-treated caspase-11 ko mice, robustly reduced tumor lesions in the lung. Instead, tumor burden of NMU-treated wild type mice that received caspase-11 ko bone marrow cells had higher tumor lesions than NMU-treated caspase-11 ko mice that received wild type bone marrow cells, strengthening what already observed for TAMs which use caspase-11/caspase-1/NLRP3 axis to promote lung tumorigenesis (11).

In support, a very interesting paper by Cheng et al. (27), similarly demonstrated the relevance of the non-hematopoietic caspase-11 in a mouse model of lung injury. However, the latter effect was mediated by TLR4. Instead, in our experimental conditions, we found that TLR4 dysfunctional mice (C3H mice) had a similar tumor burden as wild type mice, implying that caspase-11 was not induced by the non-canonical inflammasome pathway. Most likely, because we found that there was a strict correlation between caspase-4 and IL-1 α (4, 12), but not IL-1 β , release, we may speculate that caspase-4 activation and IL-1 α could be the main orchestrators of lung tumorigenesis in a non-inflammasome-dependent manner in the hematopoietic compartment. It is likely that caspase-4 in the structural cells behaves as an oncoprotein, whereas in the hematopoietic lineage it can allow IL-1 α protumorigenic activity. Indeed, the neutralization of IL-1 α in NMU-treated mice significantly reduced the levels of tumor areas than control group and, very importantly, NSCLC patients who presented high levels of IL-1 α and caspase-4 had lower median survival rate.

Lung tumor-associated caspase-4 was related to tumor cell proliferation, rather than cell death. This effect was correlated to the large subunit of caspase-4. In literature, caspase-4 and caspase-11 activity are often associated to the release of lactate dehydrogenase, LDH, as a marker of cell death. It is worldwide known that high levels of LDH characterize inflammatory patterns, together with CRP and ERS, which are highly detected in cancer patients. Similarly, in our experimental conditions, we found that all

patients positive for tumor-associated caspase-4 had high levels of LDH, which was not a measure of cell death as reported in *in vitro* assays (28, 29), but of cancer progression (30). To date, LDH is an enzyme that catalyzes the pyruvate to lactate during anaerobic conditions. The metabolomic profile of cancer patients is well-described as altered in that to promote the accumulation of pyruvate which leads to both the anaerobic (LDH-dependent) and tricyclic acid (TCA) pathway according to the tumor cell metabolic needs (31, 32). Therefore, tumor-associated as well as circulating caspase-4 (12) suggest that the inflammatory pattern in tumor cells alters the metabolomic profile to favor cell proliferation rather than cell death, as we recently demonstrated (30). In support, Trinidad et al., proved that the activation of the pyruvate kinase 4, which phosphorylates the pyruvate at the last step of glycolysis, is correlated to the metabolic phenotype of K-Ras in favor of tumor cell proliferation (33). The oncogenic K-Ras was associated with higher levels of hexokinase 2 (HK2), involved in high-rate metabolism of the glucose in lung cancer-associated TCA according to higher consumption of glutamine (34).

Similarly, various studies have demonstrated that c-MyC-driven tumors display increased glucose uptake and catabolism to lactate and TCA cycle intermediates (35). In this context we found that 85.2% of NSCLC patients who presented K-Ras mutation and 66.7% of patients who overexpressed c-MyC were positive for tissue caspase-4. The median survival rate of these two subgroups of patients was 0.97 (Figure 3J, black line) and 1 (Figure 3G, black line) year. However, our study had a limited number of patients at stage III due to the fact that these patients usually undergo therapeutic treatment without surgical resection. In addition, we found that K-ras^{LA1} and K-ras^{LA1/p53R172HΔ} Tg mice had higher lung levels of the cleaved form of caspase-11 (Figure 4D), associated to higher tumor lesions developed in double Tg mice as reported by Riccardo et al (23). These data together with the *in vitro* studies (Figure 5) suggest that caspase-4 underlies K-Ras-mediated cell proliferation. However, it has to be pointed out that Caspase-4+ K-Ras -- (Figure 3J, blue line; Figure 3L, 14.8%) or Caspase-4+ cMyC- (Figure 3G, blue line; Figure 3I, 33.3%) NSCLC patients still had lower survival rate. In this regard, a recent manuscript demonstrated that caspase-4 can lead to epithelial-mesenchymal transition in lung cancer (36), further highlighting the importance of our discovery on the role of caspase-4 in lung carcinogenesis in humans.

Another important issue is that we found that among PD-L1 negative patients, 88.1% were positive to caspase-4 (Figure 3C). Similarly, 89.8% of NSCLC patients who did not present EGFR mutation or ALK, ROS1, MET genetic alterations were positive to caspase-4. Key established predictive biomarkers for target therapy include ALK and ROS1 rearrangements, EGFR mutations, BRAF V600E point mutations, and PD-L1 expression levels (2). The National Comprehensive Cancer Network (NCCN) panel recommends testing for these key established biomarkers in patients with NSCLC to decide for the pharmacological treatment, because effective targeted therapies or immunotherapy are available. In particular, tyrosine kinase inhibitors (TKIs) and monoclonal antibodies against PD-1/PD-L1 axis are becoming the first-line therapeutic options for NSCLC patients positive to these targets. However, patients treated with TKIs develop drug resistance after around 12-15 months of treatment. Similarly, patients treated with anti-PD-1/PD-L1 monoclonal antibodies can develop resistance with various mechanisms (2). Even worse is the situation of non-mutated NSCLC patients whose sole therapeutic option could be

the classical chemotherapy or chemotherapy in addition to anti-PD-L1 antibodies, although the low expression of PD-L1 in tumor tissues. Therefore, here we identified a novel subpopulation of NSCLC patients as caspase-4 positive among which we identified patients as double positive for caspase-4 and K-Ras or c-MyC, and triple positive, identified as caspase-4, K-Ras and c-MyC positive.

Conclusions

Our study 1. highlights a novel diagnostic and prognostic oncoprotein for a subgroup of NSCLC patients; 2. identifies a subpopulation of NSCLC patients who are caspase-4 positive among non-mutated and K-Ras-mutated and cMyC overexpressing patients; 3. identifies a potential molecular mechanism between caspase-4 and K-Ras/c-MyC in the tumor mass; 4. opens new therapeutic perspectives for patients who can only be cured with the classical chemotherapy since K-Ras and cMyC are still undruggable. Therefore, the identification of caspase-4 as a novel oncoprotein could introduce novelty in the clinical yet unmet needs for NSCLC patients.

List Of Abbreviations

Non-small cell lung cancer= NSCLC

Programmed death-1= PD-1

Programmed death ligand-1= PD-L1

Epidermal growth factor receptor=EGFR

Anaplastic lymphoma kinase=ALK

Interleukin-1=IL-1

C-reactive protein=CRP

Erythrocyte sedimentation rate=ESR

High Mobility Group Box 1=HMGB1

Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat And Pyrin Domain Containing 3=NLRP3

Tumor-associated macrophages=TAMs

ROS Proto-Oncogene 1=ROS1

Met proto-oncogene=MET

Knockout= ko

N-methyl-N-nitroso-urea=NMU

Broncho-alveolar lavage fluid=BAL

tissue microarray=TMA

Carboxyfluorescein diacetate succinimidyl ester=CFSE

CASP4= caspase-4 gene

Receiver operating characteristic analysis= ROC analysis

Declarations

Ethic approval and consent to participate

Human samples in this study were obtained by patients diagnosed of operable NSCLC, undergoing surgical resection at Ospedale dei Colli, AORN, Monaldi, Naples, Italy, during the period 2014-2017. The project was approved by the institutional review board and by the Ethical Committee (approval number for lung cancer patients 1254/2014).

All animal experiments were performed under protocols that followed the Italian and European Community Council for Animal Care (2010/63/EU). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Salerno and by National Institutes of Health with the approval number 13786/2014.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests.

The authors declare that they have no competing interests.

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Authors' Contributions.

MT, CC performed the majority of the experiments; IDR, PS, CC, LP, RS (Salvi) recruited NSCLC patients; FZM and GB performed TMA histological analyses; RPA revised the submitted version of the manuscript; AP and RS designed the experiments, interpreted data, wrote the manuscript. All authors read and approved the final manuscript.

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Authors' information

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Figures

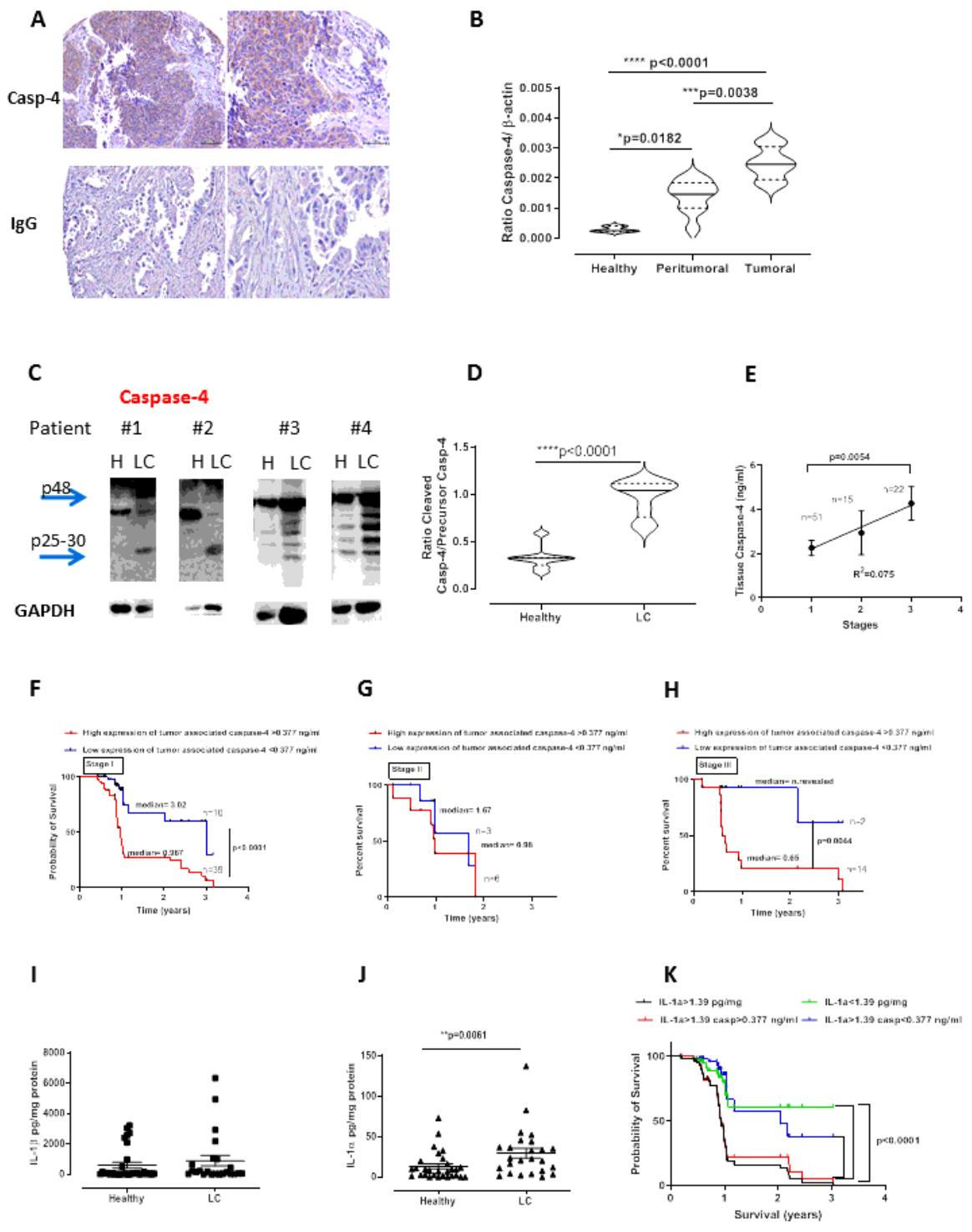


Figure 1

Figure 1

Caspase-4 positive NSCLC patients show poor survival rate. Higher levels of tumor caspase-4 were detected histologically ($n=89$) (A), by means of RT-PCR ($n=8$) (B) and western blotting ($n=9$) (C). Representative blots are shown for immunohistochemistry (A). Healthy tissues represent the non-

cancerous tissues obtained after surgical resection of tumor areas. D) Quantitative analysis of western blotting data. The levels of caspase-4 were related to the TNM stage (E) of NSCLC patients. F) Survival rate of stage I NSCLC patients who presented > or < levels of caspase-4 according to the cut-off=0.377 ng/ml; G) Survival rate of stage II NSCLC patients who presented > or < levels of caspase-4 according to the cut-off=0.377 ng/ml; H) Survival rate of stage III NSCLC patients who presented > or < levels of caspase-4 according to the cut-off=0.377 ng/ml. Levels of IL-1 β (I) and IL-1 α (J) in lung homogenates deriving by non-cancerous (healthy) and cancerous (LC) tissues. K) Survival rate of NSCLC patients according to IL-1 α and caspase-4 levels (n=61). Data are expressed as median \pm quartile range and represented as violin plots. One Way ANOVA followed by Bonferroni's post-test was applied to Figure B. Mann Whitney test was applied to Figure D, I and J; Two-Way ANOVA was applied to Figure E. Log-rank Mantel-Cox and Wilcoxon test were performed to statistically analyze the survival rate between the four groups.

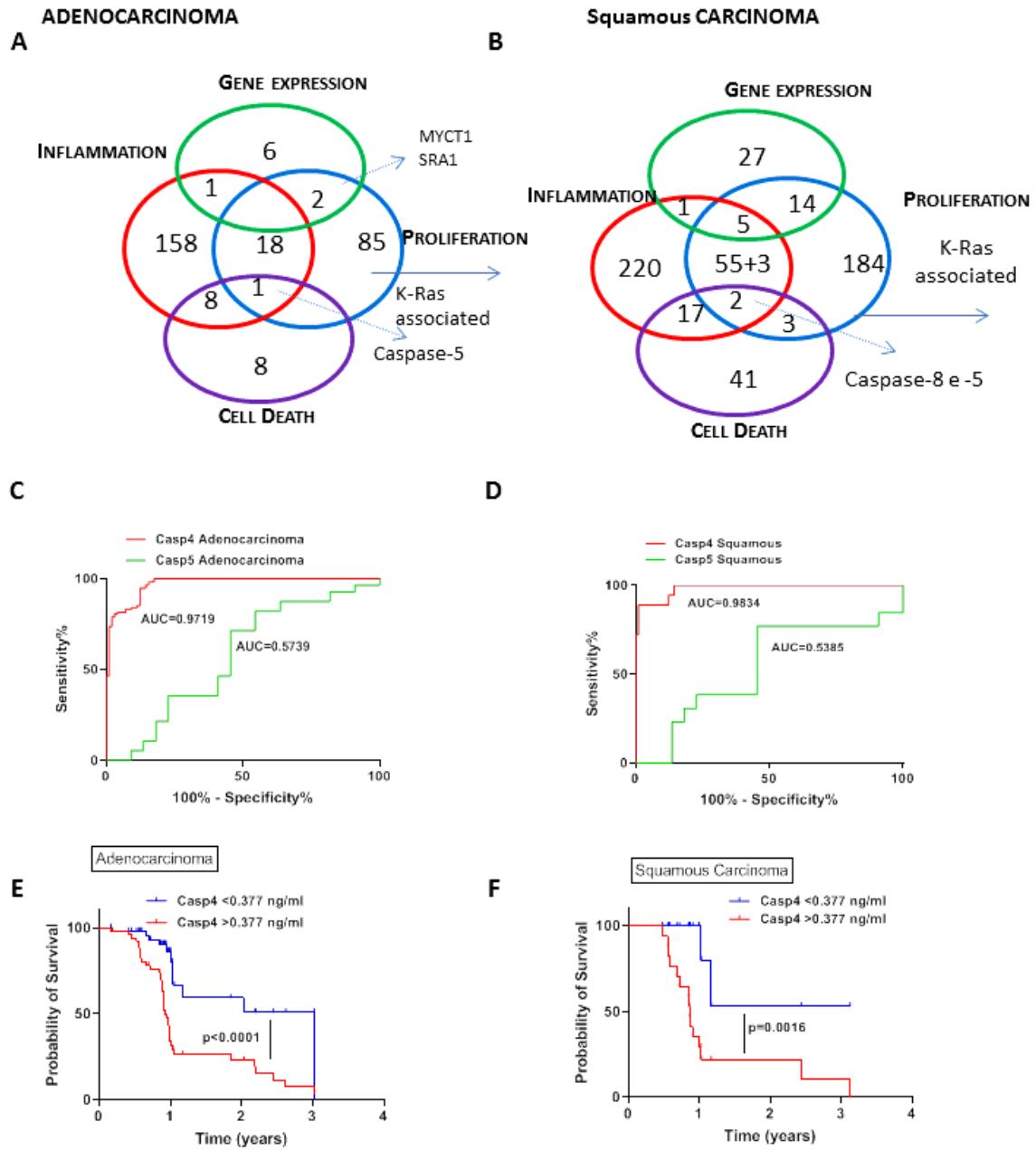


Figure 2

Figure 2

Poor survival rate of adenocarcinoma and squamous lung cancer patients according to the levels of tumor-tissue caspase-4. A and B) Venn's diagrams obtained by the analysis of genomic alteration on CASP4 (mutation or amplification or higher gene copy number) correlated to other genes involved cell death, inflammation, gene expression and cell proliferation in adenocarcinoma (A) and squamous (B) lung cancer patients by using a public database (www.cbioportal.org). Common genes were related to

KRAS, CMYC and CASP5. ROC analyses for caspase-4 and caspase-5 analyzed in samples obtained by adenocarcinoma (n=66) (C) and squamous (n=13) (D) patients. Tumor levels of caspase-4 and caspase-5 analysed by means of ELISA were correlated to the available survival rate of E) adenocarcinoma (n=53) and D) squamous (n=17) NSCLC patients. Median survival of adenocarcinoma patients with high of caspase-4 expression (E, red line) was 0.925 years vs median survival of patients with lower expression (blue line, 3.028 years). Median survival of squamous carcinoma patients with high expression of caspase-4 (F, red line) was 0.864 years vs median survival of patients with lower expression (blue line, not defined). Log-rank Mantel-Cox and Wilcoxon test were performed to statistically analyse the survival rate between the groups.

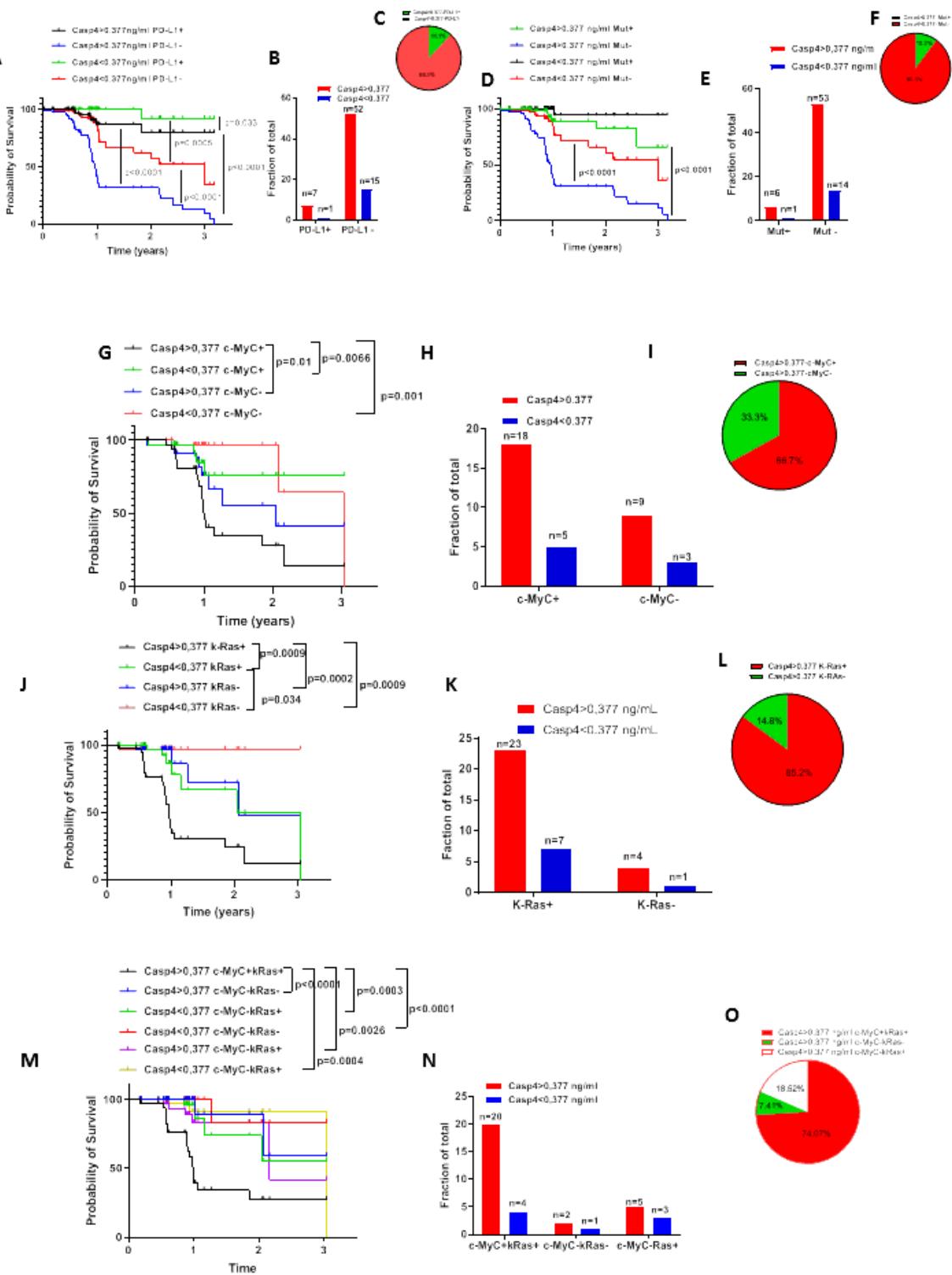


Figure 3

Figure 3

Triple positive Caspase-4, K-Ras and c-Myc NSCLC patients have poor survival rate. NSCLC patients were stratified according to the levels of caspase-4 related to PD-L1 expression (A), gene mutation (i.e. EGFR mutation, ALK or MET or ROS1 translocation) (B), or c-Myc overexpression (G, M) and K-Ras mutations (J, M). In addition, fraction of total was analysed (B, E, H, K, N) to better highlight the fraction of analysed patients according to the levels of tumor tissue caspase-4 based on a cut-off (0.377 ng/ml) obtained by

ROC analysis. Furthermore, the percentage of caspase-4 positive patients were related to PD-L1 expression (C), gene mutation (i.e. EGFR mutation, ALK or MET or ROS1 translocation) (F), or c-Myc overexpression (I, O) and K-Ras mutations (L, O). Log-rank Mantel-Cox and Wilcoxon test were performed to statistically analyse the survival rate between the groups.

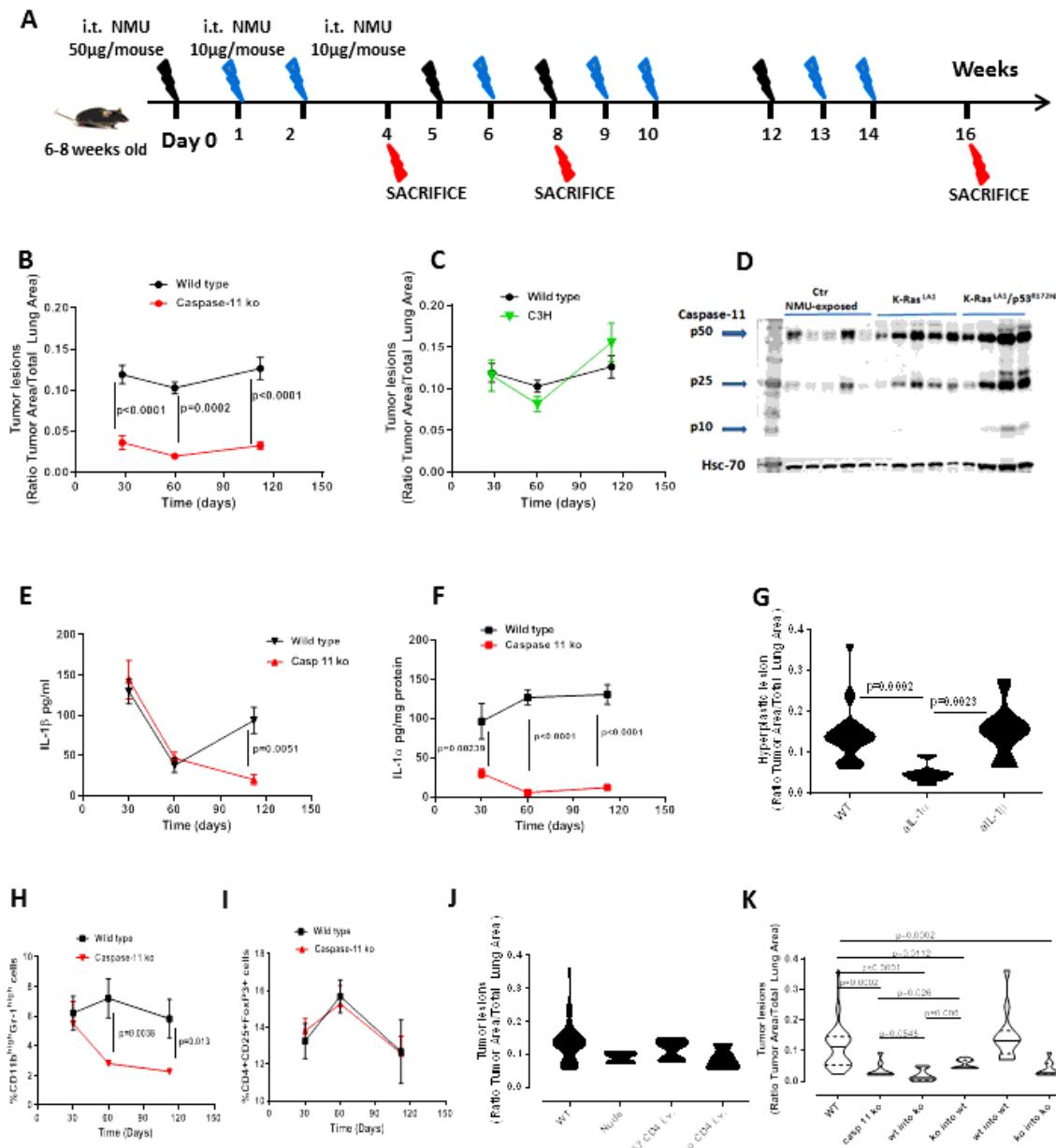


Figure 4.

Figure 4

Caspase-11 is involved in tumor progression in mice. A) Mouse model of NMU-induced carcinogenesis. B) Wild type mice (n=23) showed higher tumor lesions than caspase-11 ko (n=12) mice in a time-dependent manner. C) C3H mice (n=14) did not show any difference in terms of tumor areas than wild type mice. D) Lung tissues of K-Ras LA1 (n=10) and K-Ras LA1p53R172HB (n=10) had similar levels of cleaved caspase-11 as wild type NMU-treated C57Bl/6 mice. E) BAL levels of IL-1 β in caspase-11 ko mice (n=12) were solely reduced at longer time points compared to wild type mice (n=23); instead, F) IL-1 α levels in lung homogenates were significantly reduced at all time points in caspase-11 ko mice (n=12) compared to wild type mice (n=23) subjected to NMU. G) The neutralization of IL-1 α by means of a monoclonal antibody significantly reduced tumor hyperplastic cells in the lung of NMU-treated C57BL/6 mice. Lung immune infiltration was characterized by MDSC (H), but not by Treg (I) in NMU-treated wild type but not in caspase-11 ko mice. J) No differences in tumor area was observed in NMU-treated mice or in mice who were subjected to the adoptive transfer (n=10) of CD4+ T cells obtained by C57Bl/6 or caspase-11 ko mice. K) Bone marrow transplantation experiments showed that wt into ko mice had similar tumor area as caspase-11 ko mice treated with NMU (n=12). Data are expressed as mean \pm SEM or as median \pm quartile range (violin plots). Two-Way ANOVA followed by Sidak's multiple comparison test was applied to Figure B, C, E, F, H, I; whereas One Way ANOVA followed by Bonferroni's post test was applied to Figure G, J, K.

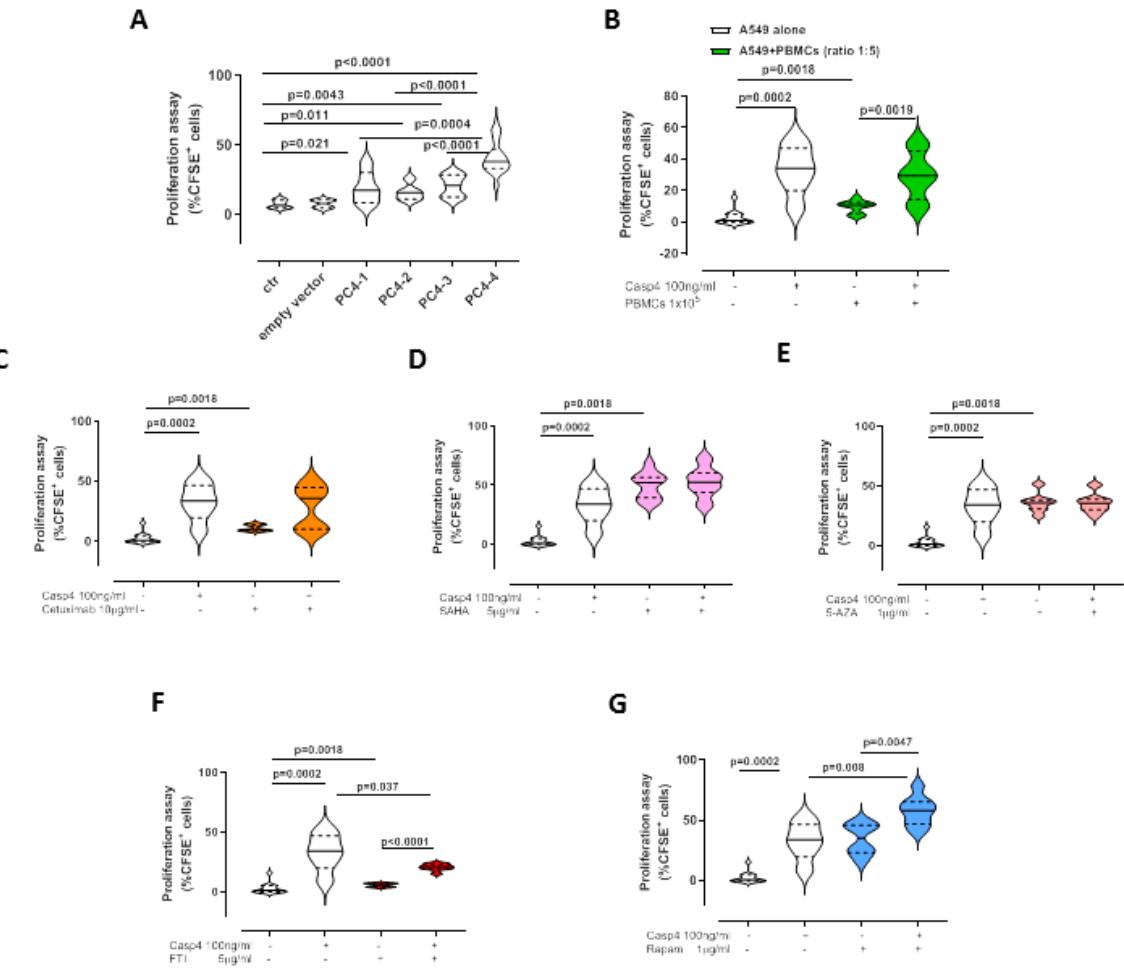


Figure 5

Figure 5

Large subunit of caspase-4 facilitates tumor cell proliferation. A549 cells were transfected with PC4-1, PC4-2, PC4-3 and PC4-4 as explained in Materials and Method section. A) Cells transfected with PC4-4 (the sole large subunit of caspase-4) showed higher CFSE positive cells than the other groups (n=15). B) Co-culture of A549 cells and PBMCs (ratio 1:5) showed similar proliferation expressed as %CFSE+ cells than A549 alone treated with the recombinant large subunit of caspase-4. The neutralization of EGFR by

means of cetuximab (C), inhibition of HDAC by means of SAHA (D), inhibition of methyltransferase (E) and mTOR (G) did not alter cell proliferation as instead observed when the inhibitor of k-RAS (FTI-276) was added (F). Experiments were performed n=15 in duplicate. Data are expressed as median± quartile range and represented as violin plots. One-Way ANOVA followed by Bonferroni's post test was applied.

Supplementary Files

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