RESEARCH PAPER

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Loss-of-function alleles of *P2RX7* and *TLR4* fail to affect the response to chemotherapy in non-small cell lung cancer

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Abbreviations: CRT, calreticulin; HMGB1, high mobility group B1; IALT, International Adjuvant Lung Cancer Trial; ICD, immunogenic cell death; IL, interleukin; LPS, lipopolysaccharide; NSCLC, non-small cell lung cancer; P2RX7, purinergic receptor P2X, ligand-gated ion channel 7; SNP, single nucleotide polymorphism; TLR4, Toll-like receptor 4; TNFα, tumor necrosis factor α; WHO, World Health Organization

The success of anticancer chemotherapy relies at least in part on the induction of an immune response against tumor cells. Thus, tumors growing on mice that lack the pattern recognition receptor TLR4 or the purinergic receptor P2RX7 fail to respond to chemotherapy with anthracyclins or oxaliplatin in conditions in which the same neoplasms growing on immunocompetent mice would do so. Similarly, the therapeutic efficacy (measured as progression-free survival) of adjuvant chemotherapy with anthracyclins is reduced in breast cancer patients bearing loss-of-function alleles of *TLR4* or *P2RX7*. *TLR4* loss-of-function alleles also have a negative impact on the therapeutic outcome of oxaliplatin in colorectal cancer patients. Here, we report that loss-of-function *TLR4* and *P2RX7* alleles do not affect overall survival in non-small cell lung cancer (NSCLC) patients, irrespective of the administration and type of chemotherapy. The intrinsic characteristics of NSCLC (which near-to-always is chemoresistant and associated with poor prognosis) and/or the type of therapy that is employed to treat this malignancy (which near-to-always is based on cisplatin) may explain why two genes that affect the immune response to dying cells fail to influence the clinical progression of NSCLC patients.

Introduction

There is ever expanding evidence suggesting that the escape from immune surveillance is a fundamental hallmark of cancer[1,2] and that therapeutic interventions with cytotoxic compounds or targeted anticancer agents are successful only when they re-establish an immune control on cancer growth.[3-5] Transplantable or primary murine cancers respond to chemotherapy with anthracyclins or oxaliplatin much more efficiently when they grow in syngenic immunocompetent mice than in immunodeficient hosts.[6,7] In line with this finding, clinical studies revealed that severe lymphopenia negatively affects the response of solid tumors to chemotherapy.[8] Moreover, while tumors growing on immunocompetent mice respond to chemotherapy with anthracyclins or oxaliplatin, tumors that grow on hosts lacking T cells, important cytokines [such as interleukin 1β (IL-1β), IL-17A and interferon γ] or their receptors continue to proliferate in an unperturbed fashion.[7,9,10] Thus, at least in some settings, immune defects are negative predictors of the response to chemotherapy.

Successful chemotherapeutics can induce a type of tumor cell death that is immunogenic,[11-13] implying that the patient's dying cancer cells function as a therapeutic vaccine, thereby eliciting an antitumor immune response that controls or eliminates the residual (chemotherapy-resistant) disease.[5,14] We have reported in the past

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that immunogenic cell death (ICD) is characterized by the pre-apoptotic exposure of calreticulin (CRT) on the cell surface,[15-17] the active secretion of ATP during the blebbing phase of apoptosis,[9,18,19] and the post-apoptotic release of the chromatin-binding non-histone protein high mobility group B1 (HMGB1).[20] CRT, ATP and HMGB1 interact with CD91, purinergic P2RX7 receptors and Toll-like receptor 4 (TLR4), respectively, on the surface of dendritic cells, thus promoting the engulfment of dying cells or their debris,[21,22] the production of IL-1β[9,23] and cross-presentation of tumor antigens to T cells,[7,24] respectively.

Mice lacking *P2rx7* or *Tlr4* phenocopy mice devoid of T cells (such as athymic *nu/nu* mice or mice injected with antibodies that deplete $CD4^+$ and $CD8^+$ T cells) in thus far that tumors growing on *P2rx7*$^{-/-}$ or *Tlr4*$^{-/-}$ mice do not respond to chemotherapy with anthracyclins or oxaliplatin in conditions in which the same neoplasms growing on normal, immunocompetent animals do so.[7,9,10,20] Similarly, adjuvant chemotherapy exhibits a reduced efficacy in patients bearing loss-of-function alleles of *P2RX7* or *TLR4*. This has been shown for patients with the single-nucleotide polymorphism (SNP) rs4986790 in *TLR4* (1307A→G; Asp299Gly; NM_138554.3:c.896A > G) and rs3751143 in *P2RX7* (1513A→C; Glu496Ala; NM_002562.4: c.1487A > C).[7,9,10,20]

The Asp299Gly substitution (corresponding to SNP rs4986790) impairs the affinity of TLR4 for lipopolysaccharide (LPS) and reportedly reduces the LPS-driven tumor necrosis factor α (TNFα) production by monocytes in vitro.[25] At a clinical level, SNP rs4986790 has been associated with a reduced frequency of chronic obstructive pulmonary disease,[26] but increased incidence of chronic sarcoidosis.[27] Operable breast cancer patients with one single lymph node invasion (but no distant metastasis) that are homozygous or heterozygous for SNP rs4986790 exhibit accelerated relapse upon anthracyclin-based chemotherapy as compared with patients bearing the wild type genotype.[7] Similarly, in a cohort of patients with colorectal cancer treated with oxaliplatin, homozygous or heterozygous carriers of SNP rs4986790 exhibited a more rapid relapse than age- and sex-matched patients bearing the wild type allele.[10] The Glu496Ala substitution (corresponding to SNP rs3751143) limits the affinity of P2RX7 for ATP and renders patient-derived, *Mycobacterium*-infected macrophages resistant against ATP-induced cell death. This is most pronounced for macrophages derived from homozygous subjects,[28] although some effect is also seen in the context of heterozygosity.[29] Subjects that carry one or two copies of SNP rs3751143 exhibit an enhanced susceptibility to extrapulmonary tuberculosis[29] and toxoplasmic retinochoroiditis,[30] confirming the functional impact of this polymorphism at the clinical level. Moreover, among breast cancer patients bearing two copies of the wild type *TLR4* allele, homozygous or heterozygous carriers of the *P2RX7* rs3751143 exhibited a more rapid relapse than age- and sex-matched patients who carried the wild type allele only.[9]

Based on these premises, we decided to evaluate the impact of the aforementioned *TLR4* and *P2RX7* alleles on the survival of patients with non-small cell lung cancer (NSCLC).

Results and Discussion

We took advantage of patient material from the phase III International Adjuvant Lung Cancer Trial (IALT), which compared cisplatin-based chemotherapy to no treatment in patients with resected stage-I-IIIA NSCLC, leading to the conclusion that adjuvant therapy can delay a substantial number of deaths,[31] at least in a subset of patients.[32,33] DNA from tumor specimens was extracted, yielding sufficient material to analyze the absence or presence (homo- or heterozygosity) of *TLR4* rs4986790 and *P2RX7* rs3751143 in 705 and 748 patients, respectively (**Table 1**). Subsequently, we compared patients that were homozygous for the wild type alleles of *TLR4* (**Fig. 1**; **Table 2**) or *P2RX7* (**Fig. 2**; **Table 2**) with those bearing one or two copies of the loss-of-function alleles, and then plotted the Kaplan-Meier survival curves for all patients included in the study (**Figs. 1A and 2A**), for patients that received chemotherapy (**Figs. 1B and 2B**) and for patients that did not (**Figs. 1C and 2C**). We found that neither SNP rs4986790 in *TLR4* nor SNP rs3751143 in *P2RX7* impact on the overall survival of NSCLC patients included in the IALT study. This held true when the entire patient population was analyzed (**Figs. 1A and 2A**), as well as upon the stratification of patients based on their allocation to chemotherapy (**Fig. 1B and C**; **Fig. 2B and C**). Stratification

Table 1. Statistical analysis of the IALT cohort

	TLR4				P2RX7			
Variable	**Wild type (n = 460)**	**Mutated (n = 245)**	**Total (n = 705)**	**Univariate p (trend test p)**	**Wild type (n = 445)**	**Mutated (n = 303)**	**Total (n = 748)**	**Univariate p (trend test p)**
	n (%)	**n (%)**	**n (%)**		**n (%)**	**n (%)**	**n (%)**	
Sex				0.48				0.97
Female	88 (19.1)	43 (17.6)	131 (18.6)		80 (18.0)	58 (19.1)	138 (18.4)	
Male	372 (80.9)	202 (82.4)	574 (81.4)		365 (82.0)	245 (80.9)	610 (81.6)	
Age (years)				*< 0.001**				0.07
< 55	150 (32.6)	69 (28.2)	219 (31.1)		122 (27.4)	103 (34.0)	225 (30.1)	(0.02)
55–64	218 (47.4)	88 (35.9)	306 (43.4)		194 (43.6)	132 (43.6)	326 (43.6)	
> 64	92 (20.0)	88 (35.9)	180 (25.5)		129 (29.0)	68 (22.4)	197 (26.3)	

Table 1. Statistical analysis of the IALT cohort (cont.)

	TLR4				P2RX7			
Variable	**Wild type (n = 460)**	**Mutated (n = 245)**	**Total (n = 705)**	**Univariate p (trend test p)**	**Wild type (n = 445)**	**Mutated (n = 303)**	**Total (n = 748)**	**Univariate p (trend test p)**
	n (%)	**n (%)**	**n (%)**		**n (%)**	**n (%)**	**n (%)**	
Stage				0.93				0.43
I	151 (32.8)	89 (36.3)	240 (34.0)	(0.71)	145 (32.6)	110 (36.3)	255 (34.1)	(0.55)
II	110 (23.9)	56 (22.9)	166 (23.5)		102 (22.9)	74 (24.4)	176 (23.5)	
III	199 (43.3)	100 (40.8)	299 (42.4)		198 (44.5)	119 (39.3)	317 (42.4)	
N of TNM				0.61				0.20
0	202 (43.9)	122 (49.8)	324 (46.0)	(0.34)	202 (45.4)	142 (46.9)	344 (46.0)	(0.83)
1	136 (29.6)	70 (28.6)	206 (29.2)		124 (27.9)	94 (31.0)	218 (29.1)	
2	122 (26.5)	53 (21.6)	175 (24.8)		119 (26.7)	67 (22.1)	186 (24.9)	
T of TNM				0.83				1.00
1	69 (15.0)	37 (15.1)	106 (15.0)	(0.68)	68 (15.3)	48 (15.8)	116 (15.5)	(0.93)
2	278 (60.4)	142 (58.0)	420 (59.6)		266 (59.8)	178 (58.7)	444 (59.4)	
3	113 (24.6)	66 (26.9)	179 (25.4)		111 (24.9)	77 (25.4)	188 (25.1)	
Histology				0.70				0.77
Adenocarcinoma	157 (34.1)	75 (30.6)	232 (32.9)		141 (31.7)	98 (32.3)	239 (32.0)	
Other NSCLC	55 (12.0)	32 (13.1)	87 (12.3)		59 (13.3)	35 (11.6)	94 (12.6)	
Squamous cell carcinoma	248 (53.9)	138 (56.3)	386 (54.8)		245 (55.1)	170 (56.1)	415 (55.5)	
Surgery				0.49				0.29
Lobe- or segmentectomy	278 (60.4)	144 (58.8)	422 (59.9)		270 (60.7)	178 (58.7)	448 (59.9)	
Pneumonectomy	182 (39.6)	101 (41.2)	283 (40.1)		175 (39.3)	125 (41.3)	300 (40.1)	
WHO PS**				0.54				0.01
0	259 (56.3)	132 (53.9)	391 (55.5)	(0.33)	220 (49.4)	19 (63.7)	413 (55.2)	*(< 0.001)*
1	163 (35.4)	93 (38.0)	256 (36.3)		182 (40.9)	94 (31.0)	276 (36.9)	
2	38 (8.3)	20 (8.2)	58 (8.2)		43 (9.7)	16 (5.3)	59 (7.9)	
Lymphoid infiltration				0.55				*0.04*
Intense	52 (11.3)	25 (10.2)	77 (10.9)		40 (9.0)	43 (14.2)	83 (11.1)	
Weak	408 (88.7)	220 (89.8)	628 (89.1)		405 (91.0)	260 (85.8)	665 (88.9)	
Pleural invasion				0.80				0.61
No	422 (91.7)	223 (91.0)	645 (91.5)		406 (91.2)	279 (92.1)	685 (91.6)	
Yes	38 (8.3)	22 (9.0)	60 (8.5)		39 (8.8)	24 (7.9)	63 (8.4)	
Vascular invasion				0.73				0.74
No	318 (69.1)	172 (70.2)	490 (69.5)		313 (70.3)	215 (71.0)	528 (70.6)	
Yes	142 (30.9)	73 (29.8)	215 (30.5)		132 (29.7)	88 (29.0)	220 (29.4)	
Lymphatic invasion				0.39				0.42
No	139 (30.2)	81 (33.1)	220 (31.2)		133 (29.9)	99 (32.7)	232 (31.0)	
Yes	321 (69.8)	164 (66.9)	485 (68.8)		312 (70.1)	204 (67.3)	516 (69.0)	
Quality after final H&E				0.30				0.98
Average	46 (10.0)	32 (13.1)	78 (11.1)		47 (10.6)	34 (11.2)	81 (10.8)	
Good	414 (90.0)	213 (86.9)	627 (88.9)		398 (89.4)	269 (88.8)	667 (89.2)	

*Significant p values are indicated in italic. p values were calculated by two-sided χ^2 tests, using logistic regressions stratified on center. **World Health Organization (WHO) scores for performance status (PS) range from 0 to 2, with score of 0 indicating no symptoms, 1 mild symptoms and 2 moderate symptoms. Abbreviations: H&E, hematoxylin and eosin; NSCLC, non small cell lung cancer; TNM, tumor node metastasis.

according to additional criteria at the level of the genotype (such as heterozygosity vs. homozygosity) or the treatment (cisplatin plus etoposide vs. cisplatin plus microtubular inhibitors) did not reveal any consistent impact of the analyzed *TLR4* or *P2RX7* polymorphisms on patient survival (not shown). Along similar lines, a combined analysis in which patients were classified into groups bearing one or several loss-of-function alleles failed to uncover any predictive or prognostic impact of *TLR4* SNP rs4986790 and *P2RX7* SNP rs3751143 (**Fig. 3**, **Table 2**). Intriguingly both *TLR4* and *P2RX7* mutational statuses were found to be significantly associated with age, and that of *P2RX7* also with World Health Organization (WHO) performance status and lymphoid infiltration (**Table 1**).

Altogether, the data presented here indicate that loss-of-function alleles in *TLR4* and *P2RX7* do not affect overall survival in NSCLC patients, irrespective of the administration and type of chemotherapy.

What may be the reasons for this finding, which clearly differs from our previous observations on anthracyclin-based adjuvant chemotherapy in breast cancer and oxaliplatin-based adjuvant chemotherapy in colorectal cancer?[5,7,9,10] There are at least two possible explanations for this discrepancy. First, the prognosis of NSCLC is intrinsically dismal,[34] and bronchial carcinomas may be subjected to a less vigorous immunosurveillance than tumors located in other organs such as the mammary gland or the colic mucosa.[35] NSCLC may also be particularly efficient in suppressing the function of local innate immune effectors such as natural killer (NK) cells,[36] or in escaping $CD8^+$ T cell-mediated adaptive immunity (for instance due to impaired expression and/or function of FAS).[37] Irrespective of this, which remains a mere matter of speculation, the literature reporting a clinical benefit from the infiltration of NSCLC by effector T cells is relatively scarce as compared with the plethora of reports demonstrating that mammary and colorectal cancers are controlled by a clinically relevant level of immunosurveillance.[5,38,39] Of note, it appears that the infiltration of the tumor mass by dendritic cells has a more positive impact on NSCLC prognosis than that by effector T cells.[40] These latter findings, together with recent results from the largest multicentric NSCLC gene profiling study to date, the NIH Director's Challenge Study,[41] support further investigations on the role of the immune system in NSCLC and on its relevance during the response to chemotherapy. Second, the standard treatment for NSCLC is based on cisplatin, a DNA damaging agent that, besides being associated with a high rate of relapse due to the development of chemoresistance,[42] induces non-immunogenic cell death.[10] This means that cells succumbing to cisplatin in vitro fail to elicit specific anticancer immune responses when inoculated in vivo.[43,44] Accordingly, the response to cisplatin of experimental cancers in vivo is not influenced by the absence or presence of *TLR4*.[10] Of note, cisplatin is usually combined with other chemotherapeutic agents that are also unable to induce ICD, such as etoposide,[15,45] or with microtubular inhibitors, whose capacity to

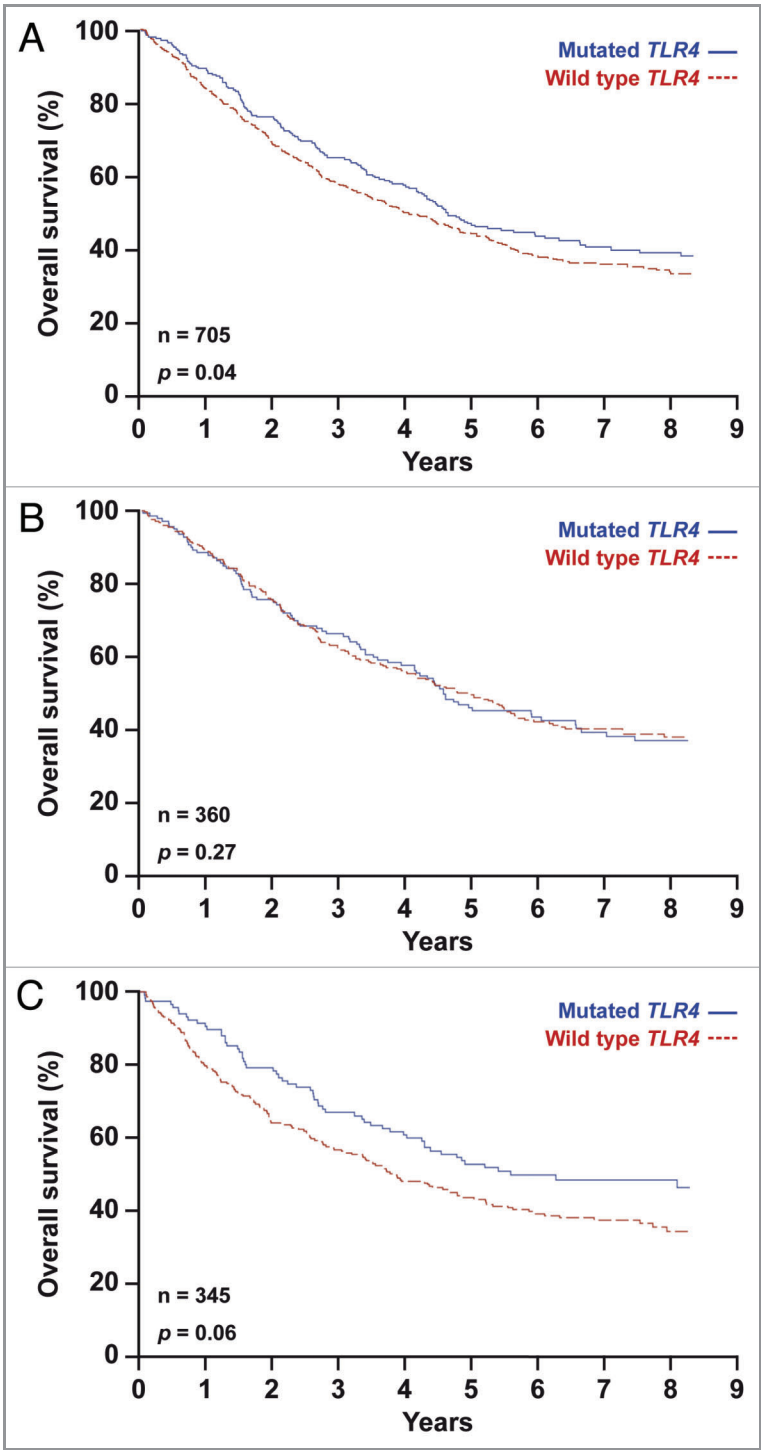

Figure 1. Kaplan-Meier estimates of overall survival in non-small cell lung carcinoma (NSCLC) patients bearing one or two copies of the *TLR4* rs4986790 SNP (mutated *TLR4*) or the wild type allele only (wild type *TLR4*). Overall survival according to *TLR4* status in all 705 patients (A), in patients subjected to chemotherapy (B) and in untreated patients (C).

Table 2. Overall survival according to treatment and mutational status

Group	All patients	Treated patients	Untreated patients
Population with wild type TLR4			
Deaths/total n° of patients	298/460	149/230	149/230
Median OS - months	4.1	4.4	3.8
Population with mutated TLR4			
Deaths/total n° of patients	144/245	85/130	59/115
Median OS - months	4.6	4.5	5.6
HR* for death (95% CI)	0.81 (0.66–0.99)	0.85 (0.64–1.13)	0.73 (053–1.01)
p value	0.04	0.27	0.06
Population with wild type P2RX7			
Deaths/total n° of patients	281/445	150/232	131/213
Median OS - months	4.4	4.6	3.9
Population with mutated P2RX7			
Deaths/total n° of patients	187/303	99/157	88/146
Median OS - months	4.5	4.3	4.7
HR* for death (95% CI)	1.03 (0.85–1.24)	1.06 (0.81–1.38)	1.00 (0.76–1.32)
p value	0.77	0.68	1.00
Population with wild type TLR4 and P2RX7			
Deaths/total n° of patients	162/241	83/124	79/117
Median OS - months	4.0	4.3	3.5
Population with at least one TLR4 or P2RX7 mutation			
Deaths/total n° of patients	263/436	145/228	118/208
Median OS - months	4.6	4.5	5.2
HR* for death (95% CI)	0.84 (0.68–1.03)	0.86 (0.65–1.15)	0.80 (0.59–1.08)
p value	0.09	0.32	0.14

*Hazard ratios (HR) reflect the comparison between the mutated and the wild type groups. All hazard ratios were adjusted for sex, age, tumor stage, histology type and the presence or absence of pleural invasion, and were stratified according to clinical center. Abbreviations: CI, confidence interval; OS, overall survival.

induce ICD has not yet been thoroughly evaluated. Importantly, our findings do not rule out a role for TLR4 and P2XR7 in the pathogenesis and response to therapy of NSCLC, as multiple epigenetic mechanisms, including promoter hypermethylation, alternative splicing and microRNA-mediated gene regulation, might be responsible for the functional inhibition of these two proteins. Future studies investigating the how the innate and cognate immune systems influence the clinical progression of NSCLC will surely provide deeper insights into the results of our retrospective analysis.

Materials and Methods

Patients and study design. One-thousand-eight-hundred-sixty-seven patients were enrolled in the IALT study, upon informed consent. The clinical features of the patient cohort can be found elsewhere.[31] Paraffin-embedded tumor blocks (obtained at the time of surgery) were collected in 14 distinct countries by 28 medical centers that participated into the IALT study with more than ten patients.[32] Approval was obtained by local institutional review boards according to the national regulations. A total of 867 samples were reviewed at the Centre Hospitalier Universitaire Albert Michallon (Grenoble, France) and histopathologically classified according to the system adopted by the World Health Organization (WHO) in 2004. The amount and quality of 822 among the 867 paraffin blocks were judged adequate for experimental procedures. Finally, 776 samples were identified as non-small cell lung cancer (NSCLC), but only 705 and 748 were available for *TLR4* and *P2RX7* genotyping, respectively.

Genotyping. Genomic DNA was isolated from paraffin-embedded tumors by means of the DNeasy Blood and Tissue Kit (Qiagen). Gene-specific Taqman® primers and genotype-specific probes (Applied Biosystems) were used to amplify a *TLR4* fragment containing the Asp299Gly single nucleotide polymorphism (rs4986790) site and a *P2RX7* fragment containing the Glu496Ala polymorphism (rs3751143). Genotypes were determined by comparing the signals from fluorescent probes (FAM and VIC) and by calculating the natural logarithm of the ratio between the FAM and VIC signals [log (FAM/VIC)].

Statistical analysis. All statistical analyses were performed with long-term survival data,[46] by means of the SAS software, version 9.2 (SAS Institute). Conditional logistic regression on an aggregate center variable was used for both univariate and multivariate analyses. Survival rates were estimated using the

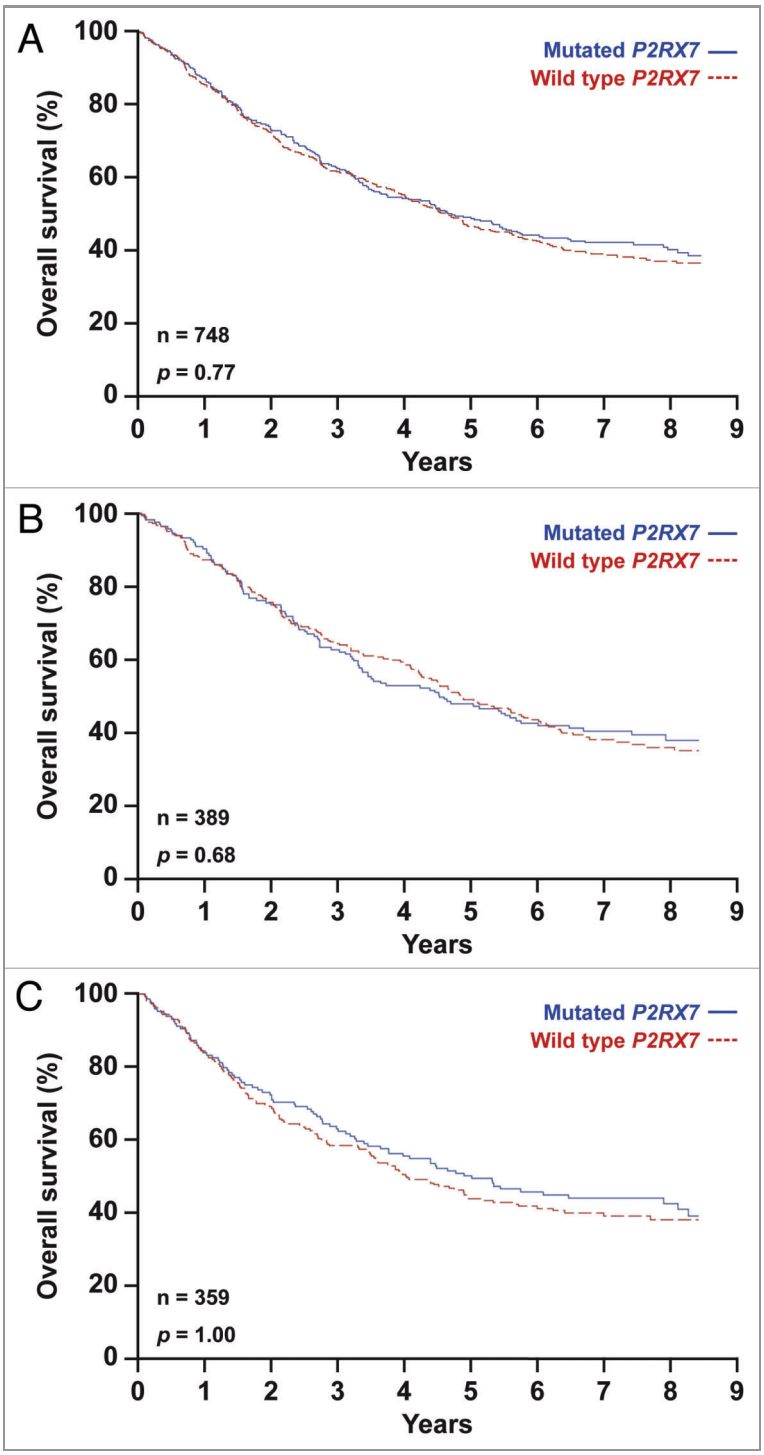

Figure 2. Kaplan-Meier estimates of overall survival in non-small cell lung carcinoma (NSCLC) patients bearing one or two copies of the *P2RX7* rs3751143 SNP (mutated *P2RX7*) or the wild type allele only (wild type *P2RX7*). Overall survival according to *P2RX7* status in all 748 patients (A), in patients subjected to chemotherapy (B) and in untreated patients (C).

Kaplan-Meier method. The prognostic values of the *TLR4* and *P2RX7* status and chemotherapy were studied using a Cox model taking into account every factor used in the stratified random assignment (center, tumor stage, type of surgery) plus clinical and histological prognostic factors (age, sex, WHO performance status, nodal status, lymphoid infiltration and revised histopathological type), as in previous IALT-based studies,[32] as well as all factors that were statistically related to the biomarker status in the multivariate logistic model ($p < 0.05$). All reported p values were two sided and only p values < 0.001 were considered statistically significant, to minimize the risk of false-positive results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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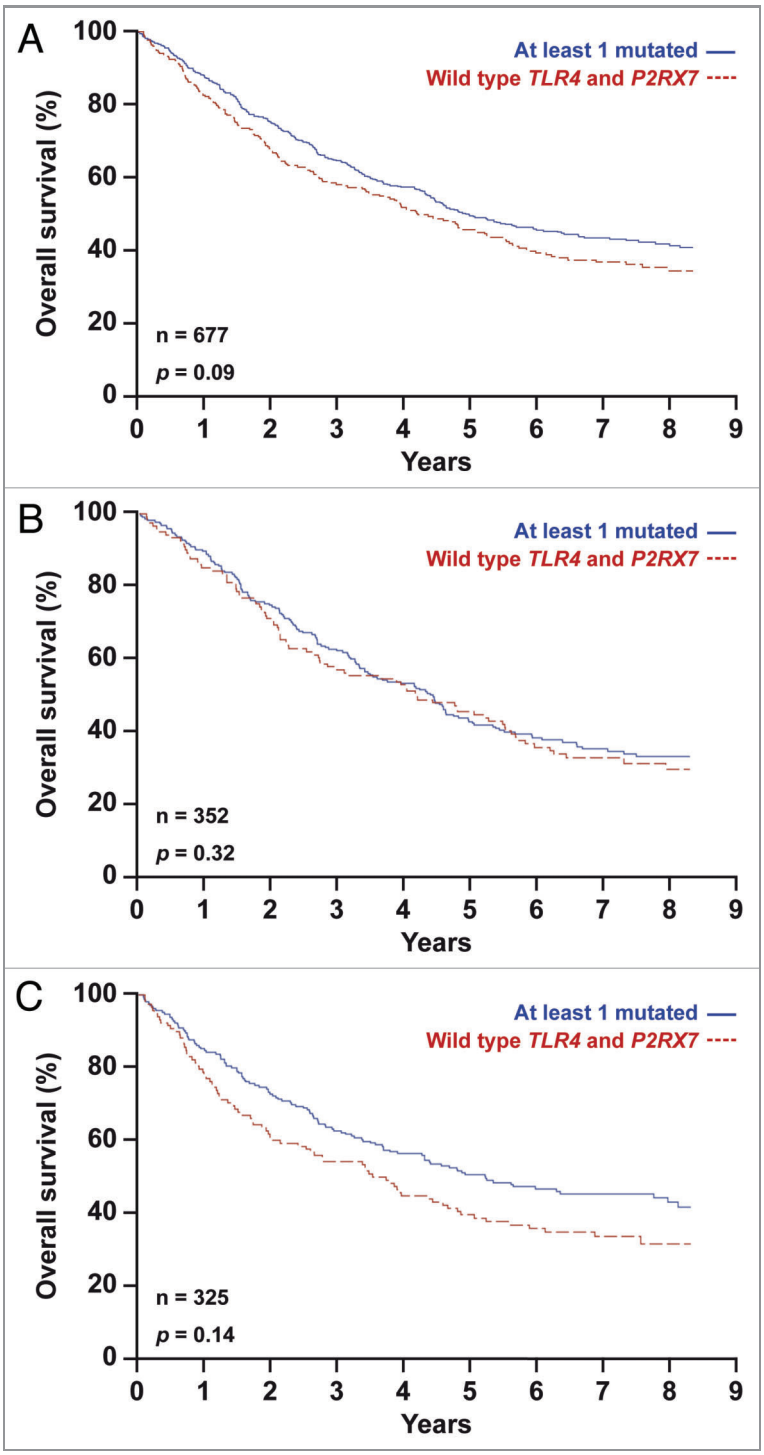

Figure 3. Kaplan-Meier estimates of overall survival in non-small cell lung carcinoma (NSCLC) patients bearing the wild type alleles for both *TLR4* and *P2RX7* (wild type *TLR4* and *P2RX7*) or at least one variant for either *TLR4* or *P2RX7*. Overall survival according to *TLR4* and *P2RX7* status in all 677 patients (A), in patients subjected to chemotherapy (B) and in untreated patients (C).

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