

ORIGINAL ARTICLE

Validation of the diagnosis of mesothelioma and BAP1 protein expression in a cohort of asbestos textile workers from Northern Italy

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Background: Diagnosis of mesothelioma based on death certificate is subject to misclassification, which may bias the results of epidemiology studies. A high proportion of mesothelioma harbor mutations in the *BRCA1-associated protein 1 (BAP1)* gene.

Methods: We searched medical and pathology records and specimens for 127 workers from a textile-asbestos factory in Italy who died during 1963–2013 with a diagnosis of pleural or peritoneal neoplasm or mesothelioma on death certificate, to confirm the diagnosis with immunohistochemistry markers. We calculated the odds ratio of confirmation by selected characteristics and asbestos exposure variables. When sufficient pathology material was available, we analyzed BAP1 protein expression.

Results: The diagnosis of mesothelioma was histologically confirmed for 35 cases (27.6%); 5 cases were classified as non-mesothelioma (3.9%), for 33 cases a mention of mesothelioma was found on record but no sufficient material was available for revision (26.0%); no records were available for 54 cases (death-certificate-only 42.5%). Diagnostic confirmation was not associated with sex, location of the neoplasm, age, or duration of employment; however, there was a significant association with time since first employment (*P* for linear trend 0.04). An association between duration of employment and time since first employment was observed for confirmed cases but not for death-certificate-only cases. BAP1 protein was lost in 18/35 cases (51.4%), without an association with sex, location, age, indices of asbestos exposure, or survival.

Conclusions: We were able to confirm by immunohistochemistry a small proportion of mesothelioma diagnoses on certificates of deceased asbestos workers, and confirmation correlated with latency of asbestos exposure but not other characteristics. BAP1 protein loss is a frequent event in mesothelioma of asbestos-exposed workers, but does not correlate with exposure.

Key words: mesothelioma, asbestos exposure, death certificates, *BRCA1-associated protein 1*, diagnostic confirmation

Introduction

We recently reported the results of the mortality analysis of a cohort of former workers of an asbestos textile factory in Northern Italy [1]. These workers experienced very high exposure to asbestos, including crocidolite, which resulted in a high number of deaths from pleural and peritoneal mesothelioma.

Death certificates have been traditionally used to identify cases of malignant neoplasms in occupational studies, but it is known that this approach entails some degree of misclassification [2].

This issue is particularly relevant for malignant mesothelioma. On the one hand, mesothelioma can be misdiagnosed as primary or secondary lung cancer, or be ignored all together (false negatives). On the other hand, other primary or secondary thoracic neoplasms, mainly peripheral lung cancer, can be wrongly diagnosed as mesothelioma (false positives): this problem may be exacerbated if the person in charge of compiling the cause of death on the certificate has knowledge of previous asbestos exposure of the decedent [3].

Several authors have compared the diagnosis of mesothelioma (or pleural/peritoneal tumor) on death certificates with medical records or pathology reports, including autopsy [4–12]. These studies reported specificity and sensitivity of death certificate-based diagnoses in the range 40%–85%, and, in studies considering multiple types of cancer, both specificity and sensitivity were lower for mesothelioma than for other cancers, in particular lung cancer. In general, older studies reported lower sensitivity, peritoneal mesothelioma was more frequently misclassified than pleural, and there was a tendency toward lower reliability of such certificates with increasing age at death. With a few exceptions (see Ref. [9]), these studies were based on a relatively small number of deaths, and the validity of death certificates was not associated with indicators of asbestos exposure.

BRCA1-associated protein 1 (BAP1) is a nuclear deubiquitinating enzyme that acts as a tumor suppressor gene and plays a role in various cellular processes [13–16]. *BAP1* gene is located on chromosome 3p21, a region that harbors both germline and somatic gene alterations identified in both hereditary and sporadic mesothelioma [14, 17–20]. Alteration of *BAP1* at genetic level, independently of the underlying mechanism (e.g. gene deletion or insertion, point mutation, gain, or loss), translates into nuclear loss of *BAP1* expression at protein level, identifiable by immunohistochemistry (IHC) with high specificity [18, 20, 21]. Furthermore, emerging evidence supports a role of *BAP1* gene alteration to enhanced cancer susceptibility. Kadariya et al. [16] have recently shown in mouse models that there is a possible gene–environment interaction between *BAP1* mutations and asbestos exposure in the development of this disease. In this context, Napolitano et al. [22] found that in mice carrying heterozygous germline *BAP1* mutations mesothelioma develops upon exposure to asbestos levels that rarely cause the disease in wild-type mice.

In this analysis based on the cohort of Italian asbestos cohort [1], we aimed at (i) evaluating the odds ratio (OR) of diagnostic confirmation according to selected variables; (ii) evaluating the mortality rate ratio (MRR) for confirmed cases versus non-confirmed cases, according to several indices of asbestos exposure; and (iii) in the subset of confirmed cases, evaluating the association between selected variables and the expression of BAP1 protein, whose gene mutation in mice is associated with higher susceptibility to mesothelioma upon low levels of exposure to asbestos.

Methods

The study population has been described in previous reports [1, 23, 24] and includes 1083 women and 894 men who had worked in an asbestos textile factory sited in the metropolitan area of Turin in Piedmont, Northern Italy, between 1946 and 1984. The main type of asbestos used in the plant was chrysotile, but crocidolite was also present; the cohort was defined based on employment records obtained from personnel records at the factory. Several time-related exposure variables were calculated, including time since first employment, duration of employment, and time since last employment. Vital status and causes of death were ascertained up to 2013 through population registers and death certificates from local authorities. A total of 127 cohort members were reported dead between 1963 and 2013 with a diagnosis of pleural or peritoneal cancer (or mesothelioma) on death certificate, 108 of them were included in the most recent mortality analysis, which was restricted to 1977

workers who were employed after 1946 [1], while for 11 decedents the diagnosis of pleural or peritoneal cancer or mesothelioma was reported among the contributory causes of death, and 8 did not satisfy the inclusion criteria of the mortality, as they had not worked in the asbestos textile factory after 1946.

As first step of the validation study, we sought medical or pathology records of all 127 cohort members with a diagnosis of pleural or peritoneal cancer or mesothelioma on the death certificate. We checked the archives of the pathology laboratories in the main hospitals of the metropolitan area of Turin. For subjects who died in different areas, we sought information from the main hospitals of the respective areas, as reported on the death certificates. Finally we contacted the regional mesothelioma registries [25], and obtained information on the cases registered in the registry of the Piedmont region.

We abstracted the pathology and clinical information according to a standard form, including date of diagnosis, histologic type, and results of IHC tests, if available. Abstraction was made blind of indicators of asbestos exposure. For decedents with a mention of mesothelioma on the pathology or medical records, we sought to obtain a pathology specimen (typically a formalin-fixed, paraffin-embedded sample from effusion or biopsy or surgery, or slides) for pathology review and BAP1 protein expression analysis.

Morphological and IHC revision of the specimens was made by two pathologists (LR, MP) according to established guidelines [26]. Further IHC stains were carried out in those cases that did not meet the guidelines (at least two positive and two negative mesothelioma markers, [26]). Briefly, 5- μ m-thick sections from paraffin blocks were processed using an automated immunostainer (Ventana BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ, USA or Omnis Immunostainer, Dako, Glostrup, Denmark) with the following primary antibody, when applicable: Calretinin (1 : 100, rabbit polyclonal #RB-9002-R7, Thermo Fisher Scientific, Waltham, MA), Wilms tumor-1 antigen (WT1, 1 : 10, mouse clone 6FH2, Thermo Fisher Scientific), Cytokeratin 5 (1 : 100, mouse clone D5, ImPath, Menarini Diagnostics, Bagno a Ripoli, Italy), Podoplanin (1 : 150; mouse clone D2-40, Dako/Agilent, Glostrup, Denmark), Pancytokeratin (1 : 500, mouse clone AE1/AE3, Dako), epithelial membrane antigen (1 : 6000, mouse clone E29, Dako), Carcinoembryonic antigen (CEA, 1 : 15 000, rabbit polyclonal IR52661-2, Dako), TTF1 (8G7G3/1, Dako) and BAP1 (1 : 100, rabbit clone-C4, Santa-Cruz Biotechnology, Santa Cruz, CA). IHC reactions were considered positive when a weak-to-strong nuclear, cytoplasmic or membrane staining was shown. All reactions were validated by internal or external control, in particular, for BAP1 IHC a complete absence of nuclear staining was considered true negative in the presence of nuclear-positive non-neoplastic cells, such as vascular endothelium or inflammatory cells, that acted as internal positive controls.

At the end of the pathology revision, study subjects were divided into four groups: (i) *IHC-confirmed mesothelioma* (positive after histologic review and IHC staining; these cases were analyzed for BAP1 protein expression); (ii) *non-mesothelioma* (either diagnosis other than mesothelioma on pathology or medical records, or lack of confirmation after histology review and/or IHC staining); (iii) *record-based mesothelioma* (diagnosis of mesothelioma from pathology or medical records, without sufficient material for histologic review and IHC staining); (iv) *death-certificate-only mesothelioma* (no medical or pathology records; diagnosis rests on death certificate).

We conducted several statistical analyses. First, we estimated the ORs of diagnostic confirmation after review [i.e. group (i) above versus all other groups] according to demographic and clinical characteristics (sex, age at death, calendar period of death, pleural versus peritoneal location), as well as selected indices of asbestos exposure (time since first exposure, time since cessation of exposure, duration of exposure). We used the same cut points for the categories of the indices of exposure as in our original publication on mortality [1]. Sensitivity analyses were conducted with different group comparisons, e.g. group (i) versus groups (iii) and (iv), excluding group (ii).

Second, we repeated the recently published mortality analysis [1] by calculating MRRs for each of the four groups of decedents described above (decedents from pleural or peritoneal cancer belonging to the other groups were excluded from this analysis). This analysis was restricted to the 108 decedents included in the original mortality analysis; in particular, it excluded deaths and person-years above 85 years of age.

Third, in an analysis restricted to confirmed cases with BAP1 protein loss of expression, we estimated the OR of carrying a BAP1 protein loss according to demographic, clinical and exposure variables. Finally, we conducted an analysis of survival of confirmed cases of mesothelioma according to BAP1 protein status.

Multivariable logistic regression models were fit to estimate ORs after adjustment for sex, age at death, pleural/peritoneal location, duration of employment, and time since first employment. MRRs were calculated by fitting Poisson regression models [27], adjusted for sex and age. Age and the exposure variables were introduced in the Poisson regression models as time-varying covariates; age was parameterized in the models as age + age². To assess the presence of linear trends across levels of ordinal variables, we evaluated the Wald χ^2 statistic after fitting regression models including a linear term for the covariate of interest. Survival analyses were conducted by calculating Kaplan–Meier estimators and by fitting Cox regression models including the same factors as the logistic models. The STATA and SAS statistical packages were used [28, 29].

Results

Supplementary Figure S1, available at *Annals of Oncology* online, illustrates the process to validate the causes of death and to obtain and review pathology specimens. Out of the 127 decedents, no sufficient information was obtained for 54 (death-certificate-only mesothelioma, 42.5%); among the other cases, pathology samples adequate for review were obtained from 35 of them, and the diagnosis of mesothelioma was confirmed in all (IHC-confirmed mesothelioma, 27.6%); a total of 5 cases were classified as non-mesothelioma (3.9%) due to a diagnosis other than mesothelioma on medical or pathology report for four cases (i.e. 1 adenocarcinoma, 1 ovarian carcinoma, 1 peritoneal carcinoma; 1 pleurisy), and to the information received from the regional mesothelioma registry for 1 case; for the remaining 33 cases a mention of mesothelioma was found on a medical or pathology report but sufficient material for the revision was not available: these cases were classified as record-based mesothelioma (26.0%).

In particular, out of the 35 samples which were reviewed 30 were histological samples (16 from the pleura and 14 from the peritoneum) and 5 were cytological samples (2 pleural and 3 peritoneal).

Regarding histotype of the cases with histological sample, 22 were epithelioid (12 peritoneal and 10 pleural), 7 biphasic (2 peritoneal and 5 pleural) and 1 sarcomatous pleural mesothelioma.

The dates of death of IHC-confirmed mesothelioma cases were more recent than those of other groups of cases; in particular, there were no IHC-confirmed cases among the 31 cases who died before 1993, and only 6 cases among the 30 who died between 1993 and 1999.

A total of 76 of the 127 cases were also listed in the Regional Mesothelioma Registry of Piedmont: among them 56 were classified by the Registry as certain mesothelioma, 19 as probable or possible mesothelioma, and 1 as non-mesothelioma. If we consider our diagnostic validation as gold standard, the sensitivity of the classification of the Registry (certain confirmed

mesothelioma versus other) was 83% and the specificity 34% (results not shown in detail).

In the univariate analysis, there was no difference in the proportion of IHC-confirmed cases between pleural and peritoneal cases, or according to age at death, while the proportion of IHC-confirmed cases was higher among women (30.9%) compared with men (17.7%, *P*-value of difference = 0.002; results not shown in detail).

The results of the multivariate logistic regression analysis are reported in Table 1. There was no association between IHC-based diagnostic confirmation and sex, site of the neoplasm, age, or duration of employment; however, there was a significant association with time since first employment. The exclusion of cases whose confirmation was based on cytologic samples has very limited impact on the results (not shown in detail). Additional analyses based on different strategies (e.g. excluding the five cases confirmed as non-mesothelioma) provided similar results (not shown in detail).

The results of the internal analysis of indicators of asbestos exposure are reported in Table 2. The number of confirmed non-mesothelioma cases was too small to be analyzed separately. There was an association between duration of employment and time since first employment and IHC-confirmed mesothelioma, while this association was not present for death-certificate-only mesothelioma.

Table 1. Odds ratios of diagnostic confirmation

Variable	N ^a	OR	95% CI	P trend
Sex				
Male	9/32	1.00	Ref.	
Female	26/60	0.95	0.35–2.60	
Site				
Pleura	17/50	1.00	Ref.	
Peritoneum	18/42	0.69	0.27–1.78	
Age at death (years)				0.28
<60	15/35	1.00	Ref.	
60–69	9/30	0.69	0.22–2.18	
70+	11/27	0.77	0.23–2.62	
Duration of asbestos exposure (years)				0.45
<1.0	6/27	1.00	Ref.	
1.0–2.9	7/19	1.86	0.50–6.91	
3.0–9.9	14/17	5.98	1.55–23.0	
10.0+	8/29	1.42	0.40–4.98	
Time since first asbestos exposure (years)				0.04
<25.0	1/17	1.00	Ref.	
25.0–34.9	11/31	7.69	0.81–72.9	
35.0–44.9	16/27	18.0	1.80–179	
45.0+	7/17	14.3	1.20–170	

^aNumber of IHC-confirmed/other cases.

OR, odds ratio of IHC-based confirmation, adjusted for the variables in the table; CI, confidence interval; P trend, *P*-value of test for linear trend; Ref., reference category.

Table 2. Mortality rate ratios for IHC-confirmed, record-based and death-certificate-only mesothelioma, according to selected indices of asbestos exposure

	IHC-confirmed mesothelioma (N = 31)		Record-based mesothelioma (N = 30)		Death-certificate-only mesothelioma (N = 44)	
	n	MRR (95% CI)	n	MRR (95% CI)	n	MRR (95% CI)
Age at first employment						
<30	22	1 (Ref.)	19	1 (Ref.)	27	1 (Ref.)
≥30	9	0.43 (0.19–0.99)	11	0.88 (0.40–1.94)	17	0.55 (0.28–1.05)
Duration of employment						
<1	5	1 (Ref.)	7	1 (Ref.)	15	1 (Ref.)
1–4	11	2.59 (0.90–7.48)	6	0.98 (0.33–2.91)	17	1.44 (0.72–2.90)
5–9	8	2.94 (0.96–9.03)	3	0.75 (0.19–2.92)	5	0.69 (0.25–1.90)
≥10	7	2.44 (0.77–7.73)	14	3.39 (1.36–8.45)	7	0.87 (0.35–2.15)
P trend		0.12		0.008		0.53
Time since first employment						
<30	6	1 (Ref.)	10	1 (Ref.)	18	1 (Ref.)
30–44	19	4.24 (1.45–12.39)	15	1.52 (0.61–3.77)	21	1.27 (0.63–2.55)
≥45	6	4.16 (1.02–17.00)	5	1.57 (0.43–5.74)	5	0.89 (0.29–2.77)
P trend		0.03		0.42		0.93
Time since last employment						
<30	15	1 (Ref.)	20	1 (Ref.)	26	1 (Ref.)
≥30	16	1.47 (0.67–3.21)	10	0.61 (0.27–1.37)	18	1.02 (0.53–1.97)

Non-mesothelioma cases (n = 3) were not analyzed because of small numbers. In each analysis mesothelioma cases belonging to other categories were excluded. Deaths and person-years occurring at age ≥85 years were excluded.

MRR, Mortality rate ratios adjusted for age and sex; CI, confidence interval; N, number of deaths; Ref., reference category; P trend, P-value of test for linear trend.

BAP1 IHC protein expression was investigated in the 35 IHC-confirmed cases; 18/35 (51.4%) were negative for nuclear BAP1 expression (loss of expression), thus predicting a gene alteration. These cases were distributed as follows: 5/5 cytological samples, 10/22 epithelioid mesothelioma (4/12 peritoneal and 6/10 pleural), 3/7 biphasic mesothelioma (1/2 peritoneal and 2/5 pleural), and 0/1 sarcomatous mesothelioma. The results of the multivariate logistic regression are shown in [supplementary Table S1](#), available at *Annals of Oncology* online: for none of the factors included in the analysis (sex, age at diagnosis, tumor site, duration of employment, and time since first employment) there was an association with BAP1 protein expression loss.

No difference in survival was detected according to BAP1 protein expression (Figure 1); the P-value of the Kaplan–Meier test was 0.30. This result was confirmed in a multivariate Cox regression analysis (hazard ratio for negative BAP1 expression, after adjustment for sex, site of neoplasm, age at death, duration of employment and time since first employment 1.76; 95% confidence interval 0.72–4.32).

Discussion

Our study showed that for only a relatively small proportion of deaths from mesothelioma occurring among members of a historical cohort of asbestos workers (28% in our study) is possible to confirm the diagnosis on the basis of samples retrieved from the archives of pathology laboratories. It is notable in particular that despite our search efforts, no additional information was

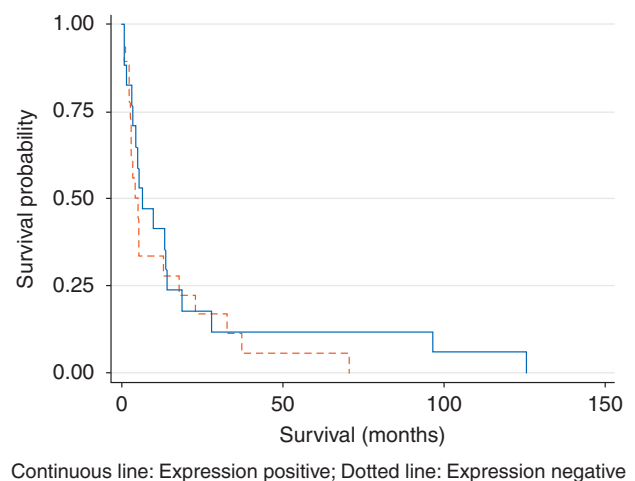


Figure 1. Survival by BAP1 protein expression.

obtained for 43% of cases in our series. These results are similar to those obtained in similar validation studies of death certificates in historical cohorts [5, 7, 9], and in part reflect the difficulty in obtaining data or pathology samples of cases who died 15 or more years ago: after excluding cohort members who died before 2000, the proportion of confirmed cases increased to 44% (29/66). In addition, our study suggests that low sensitivity in the classification of ‘certain’ confirmed mesothelioma cases may affect population-based registries of mesothelioma which do not actively seek pathologic evidence.

Our study is among the first to correlate the probability of diagnostic confirmation with clinical and environmental characteristics of the cases. No differences were found according to sex, age, and site of the mesothelioma, while time since first employment (a proxy for time since first asbestos exposure) was significantly associated a higher probability of confirmation, and duration of employment (a proxy for duration of asbestos exposure) was not. These results are consistent with the notion, supported by consistent findings from occupational epidemiology studies, that time since first exposure (i.e. latency) is the key risk factor of mesothelioma [30]. The results of the prospective mortality analysis provide support to the diagnostic validation, since the association with time since first employment is strongest among confirmed cases and absent among undetermined cases.

The analysis of BAP1 protein expression was hampered by the small number of cases with available archival material, and caution should be used in the interpretation of these results. Nonetheless, it provided some evidence of lack of association with exposure to asbestos, keeping in mind that all cases in this study were exposed to this carcinogen, and we could only differentiate them according to duration of employment or time since first employment. Other studies also failed to detect an association between asbestos exposure and either BAP1 protein expression or gene mutation [31, 32]: given the very heavy exposure experienced by workers in this cohort, our results provide novel evidence of a lack of association between asbestos exposure and BAP1 protein loss only, also in these extreme exposure circumstances. Our findings are in line with literature data that reported a higher BAP1 protein loss in epithelioid than other histotypes [21, 33] and primarily in pleural rather than peritoneal mesothelioma (55% versus 47%).

Our results of lack of difference in survival according to BAP1 protein expression are at odds with those of previous studies and meta-analyses [33–36], which suggest a survival benefit for cases harboring a genetic alteration in this gene. This may be due to the small number of cases included in our analysis.

Strengths of our study include the uniqueness of the cohort, characterized by high asbestos exposure and high risk of mesothelioma, and the extensive effort to retrieve medical records and pathology samples for the validation of diagnosis and the analysis of BAP1 protein expression. The small number of cases with sufficient material to confirm or disprove the diagnosis and to conduct the BAP1 protein analysis represents the main limitation of the study. The lack of individual data on level of exposure to asbestos is an additional limitation, which was addressed by analyzing proxy indicators such as duration of exposure.

In conclusion, our study shows that for a relatively large proportion of members of historical cohorts of asbestos workers, the information on pleural or peritoneal cancer/mesothelioma reported on death certificates cannot be validated through medical or pathology records, and even less so with review of pathology material. While the temporal and geographic features of our cohort make difficult to generalize the results to other populations, it is plausible that the general patterns we identified are applicable outside our study population.

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Disclosure

The authors have declared no conflicts of interest.

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