Introduction

Dysregulation of MYC (HGNC:7553) is an early consequence of activating mutations in APC(HGNC:583), a key driver mutation in the adenoma-carcinoma pathway in colorectal cancer (CRC). Mediated by beta-catenin via the canonical Wnt pathway, aberrant over-expression of MYC and CYCLIN D1 results in uncontrolled cellular proliferation, conferring a growth advantage to cancer cells [1,2]. The role of MYC over-expression in driving CRC tumorigenesis has been confirmed by gene expression profiling studies [3], and more recently by CRC genome-wide analysis as part of the Cancer Genome Atlas Network initiative [4].

Interestingly MYC over-expression as determined by Northern blot analysis was reported as a biomarker of good outcome in colorectal cancer as far back as 1996. [5] Currently its use as a prognostic biomarker is difficult to justify because of the limited availability and expense involved of molecular testing. Therefore testing for MYC over-expression in CRC is not currently available, feasible or warranted in the routine clinical setting.

Recently a novel highly sensitive and specific rabbit monoclonal antibody directed against the myc protein (clone Y69) has become available, feasible or warranted in the routine clinical setting, where myc immunohistochemistry in combination with bcl2 immunohistochemistry, has been used to identify the poor prognostic subgroup of non-Hodgkin lymphoma known as ‘double hit lymphoma’. [6,7] In fact MYC and BCL2 expression as determined by immunohistochemistry in formalin fixed paraffin embedded tissue has rapidly become part of the routine diagnostic assessment of patients with high grade B-cell lymphoma. [8].

We sought to revisit the use of MYC expression as biomarker in CRC by correlating outcome with MYC expression as determined by this widely available rabbit monoclonal antibody.

Materials and Methods

Our CRC cohort has been previously described. [9] Briefly, retrospective CRC cases were recruited by searching the pathology database of the Department of Anatomical Pathology, Royal North Shore Hospital, Sydney for patients who had definitive operations for CRC between 2004 and 2009. During this period this center performed centralized pathological testing for two quaternary referral hospitals with dedicated colorectal surgery units and 4 community hospitals. Therefore this patient cohort represents a true snapshot of CRC cases encountered in the Australian community as a whole.
Table 1. Clinical and pathological characteristics of 1421 consecutive CRC patients (2004–2009).

| Variable                                      | myc IHC positive | myc IHC negative | p-value* |
|-----------------------------------------------|------------------|------------------|----------|
| Gender, n (valid %)                           |                  |                  | 0.717    |
| Female                                        | 514 (52.4)       | 226 (51.2)       |          |
| Male                                          | 466 (47.6)       | 215 (48.8)       |          |
| Age at diagnosis, median (range)              | 73 (17–100)      | 75 (33–98)       | 0.071    |
| Anatomic location, n (valid %)                |                  |                  | 0.771    |
| Rectum                                       | 247 (25.5)       | 113 (25.7)       |          |
| Caecum                                       | 218 (22.5)       | 94 (21.4)        |          |
| Ascending colon                              | 142 (14.4)       | 76 (17.3)        |          |
| Transverse colon                             | 119 (12.3)       | 48 (10.9)        |          |
| Descending colon                             | 33 (3.4)         | 18 (4.1)         |          |
| Sigmoid colon                                | 211 (21.8)       | 91 (20.7)        |          |
| Histologic grade, n (valid %)                |                  |                  | 0.755    |
| Low                                          | 553 (79.0)       | 269 (80.1)       |          |
| High                                         | 147 (21.0)       | 67 (19.9)        |          |
| Lymphovascular space invasion, n (valid %)   |                  |                  | 0.663    |
| Absent                                       | 362 (36.9)       | 173 (39.2)       |          |
| Present                                      | 303 (30.9)       | 155 (35.1)       |          |
| Peritumoral lymphocyte reaction, n (valid %)  |                  |                  | 0.316    |
| Absent                                       | 34 (4.8)         | 11 (3.3)         |          |
| Present                                      | 669 (95.2)       | 326 (96.7)       |          |
| Overall Stage AJCC/TNM 7th ed, n (valid %)   |                  |                  | 0.003    |
| I                                            | 181 (18.5)       | 53 (12.0)        |          |
| IIA                                          | 298 (30.4)       | 116 (26.3)       |          |
| IIB                                          | 52 (5.3)         | 33 (7.5)         |          |
| IIC                                          | 11 (1.1)         | 4 (0.9)          |          |
| IIA                                          | 49 (5.0)         | 15 (3.4)         |          |
| IIB                                          | 237 (24.2)       | 136 (30.8)       |          |
| IIC                                          | 116 (11.8)       | 58 (13.2)        |          |
| IVA                                          | 20 (2.0)         | 12 (2.7)         |          |
| IVB                                          | 16 (1.6)         | 14 (3.2)         |          |
| MMR IHC status, n (valid %)                  |                  |                  | 0.008    |
| Proficient                                   | 770 (78.6)       | 374 (84.8)       |          |
| Deficient                                    | 210 (21.4)       | 67 (15.2)        |          |
| BRAFV600E IHC status, n (valid %)            |                  |                  | 0.035    |
| Wild type                                    | 776 (79.2)       | 371 (84.1)       |          |
| Mutant                                       | 204 (20.8)       | 70 (15.9)        |          |
| IHC phenotypes, n (valid %)                  |                  |                  | 0.001    |
| MMRp/BRAFwt                                  | 714 (72.9)       | 339 (76.9)       |          |
| MMRd/BRAFwt                                  | 61 (6.2)         | 32 (7.3)         |          |
| MMRd/BRAFV600E                               | 149 (15.2)       | 35 (7.9)         |          |
| MMRp/BRAFV600E                               | 56 (5.7)         | 35 (7.9)         |          |

*Reports on the significance of differences between myc positive and negative groups for each variable, using either Pearson chi-square test (with continuity correction for 2 x 2 tables) for categorical variables or Mann-Whitney U test for age.

doi:10.1371/journal.pone.0087456.t001

Exclusion criteria were tumors of extra-colonic and appendiceal location, tumors treated exclusively endoluminally and histological types other than adenocarcinoma defined by the WHO 2010 system. [10].

Tumors were independently reviewed by three pathologists (CT, KDS and AG) to confirm the diagnosis and to restage the tumors according to 7th edition 2009 AJCC/TNM. [11] For resections involving synchronous tumors, the tumor with the highest pathologic stage was selected and annotated. Tissue microarrays (TMA) containing duplicate 1 mm cores were then constructed from available tumor tissue blocks.
Table 2. Multivariable binary logistic regression showing adjusted effect of MMR/BRAF IHC phenotype on myc over-expression in 1421 CRCs.

| Variable                        | Multivariable analysis |
|---------------------------------|------------------------|
| Age at diagnosis                |                         |
| Overall Stage AJCC/TNM 7th edition | 0.99 (0.98–1.00), 0.02 |
| MMR/BRAF IHC phenotype          |                         |
| MMRp/BRAFV600E                   | 1.00                    |
| MMRd/BRAFV600E                   | 0.88 (0.58–1.38), 0.57 |
| MMRd/BRAFV600E                   | 2.17 (1.45–3.24), 0.01 |
| MMRp/BRAFV600E                   | 0.85 (0.54–1.34), 0.48 |

doi:10.1371/journal.pone.0087456.t002

MYC over-expression was assessed by myc IHC, scored using a two-tiered visual system by a practicing surgical pathologist (CT) blinded to all clinical, pathological and outcome information. Nuclear staining of any intensity in greater than 10% of neoplastic cells was scored as positive (over-expressed). All other patterns of staining were scored as negative. An external control (a MYC amplified lymphoma) was run with each batch of slides.

DNA mismatch repair (MMR) and BRAF IHC status were determined by immunohistochemistry as previously described [9,12].

In order to examine the accuracy of TMA IHC interpretation, Myc IHC was performed on whole sections of CRCs from 2004, interpreted by same assessor on TMA (CT) blinded to all clinical, pathological and TMA data.

Correlation between MYC over-expression and CRC patient clinicopathological variables were examined in 2×2 contingency tables for categorical variables or using the Mann-Whitney test for age (Table 1). Variables which showed a significance of p<0.10 were included in a binary logistic regression model to further examine the relationship between MYC over-expression and DNA mismatch repair (MMR)/BRAF status IHC phenotype (Table 2).

Follow-up was obtained by the examination of hospital medical records, those from surgeons’ private rooms and archival public death notices and obituaries in the state of New South Wales, Australia. Overall survival was defined as the duration alive from time of definitive surgery. Patients were followed up until death or their last date of follow-up not more than 7 years after definitive surgery.

Kaplan Meier analysis was used to report 5-year overall survivals for CRCs with and without MYC over-expression, with the difference assessed by Log Rank Test. Multivariable cox regression was used to explore the association between adjusted effect of MYC over-expression on overall survival, in a full model which included age at diagnosis, gender, anatomic site of tumor, tumor stage, combined mismatch repair deficiency and BRAF IHC status, tumor size, histologic grade, presence or absence of lymphovascular space invasion and peritumoral lymphocyte response status.

A p<0.05 was taken as significant, except in the initial modeling using binary logistic regression (mentioned above). Statistical analyses were performed using IBM SPSS Statistics v21 on OSX.

Results

A total of 1421 CRCs were assessed on TMA. The clinical and pathological features are summarized in Table 1. Briefly, there was approximately equal gender distribution, with a median age of diagnosis at 74 years (range 17–100 years). The 5-year overall survival for the cohort was 66.3%, with a mean of 52.9% (95%CI = 5.14–5.44).

Myc IHC showed consistent nuclear immuno-localisation, and there was a visible distinction between cases which were negative (scored as negative for over-expression), and those which showed weak to strong staining in 10% or more neoplastic cells (scored as positive for over-expression). Examples of myc IHC staining characteristics are presented in Figure 1. The prevalence of MYC over-expression in CRC was 69%.

The clinical and pathological features of myc IHC negative (n = 441, 31.0%) and myc IHC positive (MYC over-expressed, n = 980, 69.0%) CRCs are summarized in Table 1. MYC over-expression was significantly associated with tumor stage and MMR/BRAF IHC phenotype, but not gender, anatomic location, histologic grade, presence or absence of lymphovascular space invasion, nor peritumoral lymphocyte reaction status. The relationship between MYC over-expression and age at diagnosis tended to significance (p = 0.07).

Table 2 shows the adjusted effect of of MMR/BRAF IHC phenotype on myc IHC status. MYC over-expression was strongly associated with the MMRd/BRAFV600E IHC phenotype [odds ratio = 2.17 (95%CI = 1.45–3.24), p<0.01].

The 5-year survival for CRCs with MYC over-expression was 93.2% (overall 50th centile survival 3.06 years, interquartile range 0.56–5.22), compared with myc negative CRC of 57.3% (overall 50th centile survival 2.32 years, interquartile range 0.31–4.26).

The effect of myc status on CRC overall survival is displayed in Figure 1. CRCs with MYC over-expression showed significantly better survival compared to myc negative cases (Log Rank test p<0.01), with a crude effect (univariable model) hazard ratio of 0.67 [95%CI = 0.54–0.83], p<0.01. This crude effect on overall survival remained significant even when stratified by MMR/
BRAF IHC phenotype [hazard ratio = 0.68 (96% CI = 0.54–0.84), p = 0.01].

In the full multivariable model, the adjusted effect of MYC over-expression on overall survival became insignificant [hazard ratio = 0.91 (95% CI = 0.69–1.20), p = 0.52] due to the dominant effect of age at diagnosis, tumor stage and lymphovascular space invasion status and the MMR deficient/BRAFV600E mutant IHC phenotype on survival (Table 3). Table 4 displays the full model including interaction between myc IHC and MMR/BRAF status.

There was substantial agreement between TMA and whole section myc IHC scores [kappa = 0.742, p < 0.01]. All positive TMA cases were positive on whole sections. There were 11 discordant cases (negative on TMA, positive on whole section), all due to patchy staining. Univariable Cox regression was also performed on whole sections, confirming the improved overall survival of CRCs showing MYC over-expression (Figure 2).
Discussion

Previous studies on MYC expression in CRC have been inconsistent and conflicting presumably due to the use of small cohorts (n ranging from 38 to 310) and the use of a variety of detection methods including IHC, fluorescent in-situ hybridization (FISH), RNA-based analysis and DNA copy number. [5,13–17]. Older studies using IHC have been limited by the use of antibodies which demonstrated aberrant (cytoplasmic) immuno-localisation presumably due to poor specificity. [18]. The development and validation of a highly specific rabbit monoclonal antibody for myc has made a major impact in lymphoma diagnosis where the combination of MYC and BCL2 overexpression as determined by immunohistochemistry alone has been used to define a poor prognosis group of B-cell non Hodgkin lymphoma known as double hit lymphomas. [6,7] Interestingly, in these lymphoma studies where myc IHC and FISH assessment have resulted in conflicting results, MYC expression as determined by IHC has been a better predictor of outcome than the previous gold standard FISH. Presumably this is because MYC over-expression...
expression can be due to a variety of cellular processes rather than just simple amplification. In this context, our study, which is based on a very large and unselected cohort on 1421 CRCs including this next generation of specific rabbit monoclonal antibody, demonstrates the high prevalence of MYC over-expression in CRCs, and more importantly, the potential role of myc as a powerful prognostic biomarker in CRCs. The fact that MYC over-expression is significantly associated with better overall survival in univariable analysis is perhaps surprising given current understanding of the tumorigenic potential of MYC dysregulation [1–4]. Only one previous study has shown this effect in CRC [5]. While there is insufficient data to postulate as to the underlying mechanism, the correlation between tumorigenic potential (oncogenesis) and survival, at least for MYC, is like to be more complex than initially thought.

In this study we have chosen to examine MYC over-expression in the context of CRCs grouped according to their MMR and BRAF status, rather than MMR and BRAF status individually. It is now well established that MMR and BRAF interact in complex ways in relation to their effect on survival. For example, in MMR proficient CRCs, the presence of BRAFV600E mutation confers a worse survival, whereas in MMR deficient CRCs, BRAFV600E is a marker of the methylator phenotype, a group of CRCs which show excellent prognosis [12, 19]. Interestingly, although MYC over-expression was strongly associated with the MMRd/BRAFV600E phenotype, their lack of interaction in the full multivariable model shows that MYC’s effect on survival cannot be explained entirely just by its association with MMR/BRAF status. This suggests that the predictive effect of MYC over-expression on survival is mediated in part, by mechanisms independent of MMR/BRAF status.

One of the major drawbacks of our study is the oversimplification by which MYC over-expression is determined. The semi-quantitative nature of IHC is such that changes in IHC intensity need to be significant (in the logarithmic scale, otherwise known as the Weber-Fechner law) in order that the human eye be able to appreciate a change. This method of IHC scoring does however lend itself to more straightforward correlative analysis such as binary logistic regression, and is the predominant methodology by which most IHC is scored in the diagnostic clinical setting. Significantly, despite this drawback we were able to demonstrate significant overall survival differences on univariable analysis of myc IHC status.

Another significant drawback is the lack of disease specific outcomes and cancer specific mortality in our study cohort. Future studies employing these endpoints will provide important information which may significantly inform on clinical practice.

In summary, our study shows that myc status, as determined by IHC, is an independent predictor of survival. However, MYC over-expression on survival is not mediated entirely by its association with MMR/BRAF status, which suggests that the predictive effect of MYC over-expression on survival is mediated in part, by mechanisms independent of MMR/BRAF status.

Author Contributions
Conceived and designed the experiments: CWT AJG. Performed the experiments: CWT KDS LJ JCYC A. Clarkson LLS AJG. Analyzed the data: CWT MH A. Chou LJ AJG. Contributed reagents/materials/analysis tools: CWT KDS LJ JCYC A. Clarkson LLS AJG. Wrote the paper: CWT MH A. Chou AJG.

References
1. He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, et al. (1998) Identification of c-MYC as a Target of the APC Pathway. Science 281: 1509–1512.
2. Powell SM, Zile N, Beazer-Barclay Y, Bryan TM, Hamilton SR, et al. (1992) APC mutations occur early during colorectal tumorigenesis. Nature 359: 235–237.
3. Chan SK, Griffith OL, Tai IT, Jones SJM (2008) Meta-analysis of colorectal cancer gene expression profiling studies identifies consistently reported candidate biomarkers. Cancer Epidemiology Biomarkers & Prevention 17: 543–552.
4. Muzny DM, Bainbridge MN, Chang K, Ding HH, Drumm J, et al. (2012) Comprehensive molecular characterization of human colon and rectal cancer. Nature 487: 330–337.
5. Smith DR, Goh HS (1996) Overexpression of the c-myc proto-oncogene in colorectal carcinoma is associated with a reduced mortality that is abrogated by point mutation of the p53 tumor suppressor gene. Clin Cancer Res 2: 1049–1053.
6. Green TM, Young KH, Visco G, Xu-Monette ZY, Orazi A, et al. (2012) Immunohistochemical Double-Hit Score Is a Strong Predictor of Outcome in Patients With Diffuse Large B-Cell Lymphoma Treated With Rituximab Plus Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone. Journal of Clinical Oncology 30: 3452–3467.
7. Johnson NA, Slack GW, Savage KJ, Connors JM, Ben-Neriah S, et al. (2012) Concurrent Expression of MYC and BCL2 in Diffuse Large B-Cell Lymphoma Treated With Rituximab Plus Cyclophosphamide, Doxorubicin, Vincristine, and Prednisone. Journal of Clinical Oncology 30: 3460–3467.
8. Hu S, Xu-Monette ZY, Tzankov A, Green T, Wu L, et al. (2013) MYC/BCL2 protein coexpression contributes to the inferior survival of activated B-cell subtype of diffuse large B-cell lymphoma and demonstrates high-risk gene expression signatures: a report from The International DLBCL, Rituximab-ChOP Consortium Program. Blood 121: 4021–4031; quiz 4250.
9. Toon CW, Walsh MD, Chou A, Capper D, Clarkson A, et al. (2013) BRAFV600E immunohistochemistry facilitates universal screening of colorectal cancers for Lynch syndrome. Am J Surg Pathol 37: 1592–1602.