Supplemental Online Content

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eAppendix. Supplementary Methods

This supplemental material has been provided by the authors to give readers additional information about their work.

eAppendix. Supplementary Methods

A. Statistical Analyses Procedure and R Output

Note: This section was written using R Markdown, which weaves together R code, the resulting R output and documentation to ensure full reproducibility of data analyses. For better readability, only the key part of the R code was presented this section The complete R code (including code for data processing, data analysis, and auxiliary functions) was provided in Section B.

1. Demographic and clinical characteristics (Table 1)

We generated descriptive statistics on patients' clinical and histological characteristics. We also compared those characteristics across different levels of tumor budding (low, intermediate or high), where a Fisher's exact test was used for comparing a categorical chracteristic and ANOVA was used for comparing a continuous characteristic.

```
vars.demo <- c("Age", "Gender", "Race", "Stage", "TumorLocation",
               "BMI", "Desmoplasia", "TumorClusters", "TumorBorder", "TumorStromaRatio",
               "KMScore", "Necrosis")
vars.type <- c("C", "C", "C", "C", "C",
               "C", "C", "C", "N", "N", "N", "N")

for (var_id in 1:length(vars.demo)) {

  if(vars.type[var_id]=="C"){
    table.self <- table(data.all[ ,vars.demo[var_id]])
    table.cross <- table(data.all[ ,c(vars.demo[var_id], "TumorBudding")])
    percent.cross <- round(t(t(table.cross) / colSums(table.cross))*100,digits=1)
    fisher.result <- fisher.test(table.cross)
    cat(paste(vars.demo[var_id], ":", sep = ""))
    print(table.self)
    print(round(table.self/sum(table.self)*100, digits=1))
    cat("\n")
    cat(paste(vars.demo[var_id], " X ", "TumorBudding Table (number):", "\n", sep = ""))
    print(table.cross)
    cat(paste(vars.demo[var_id], " X ", "TumorBudding Table (percentage):", "\n", sep = ""))
    print(percent.cross)
    cat("Fisher exact test p-value:")
    cat(round(fisher.result$p.value,digits=4), "\n\n")
  } else{
    cat(paste(vars.demo[var_id], ":\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.all[ ,vars.demo[var_id]], na.rm = T), "(",
              min(data.all[ ,vars.demo[var_id]], na.rm = T), ",",
              max(data.all[ ,vars.demo[var_id]], na.rm = T), ")", sep = ""))
    fit.anova <- aov(data.all[ ,vars.demo[var_id]] ~ data.all[ ,"TumorBudding"])
    p.anova <- round(summary(fit.anova)[[1]]$Pr[1],digits=4)
    cat("\n")
    cat("ANOVA p-value:")
```

```
    cat(p.anova, "\n\n")
    data.i.tumorbudding.1 <- data.all[data.all[ ,"TumorBudding"]==1,vars.demo[var_id]]
    data.i.tumorbudding.2 <- data.all[data.all[ ,"TumorBudding"]==2,vars.demo[var_id]]
    data.i.tumorbudding.3 <- data.all[data.all[ ,"TumorBudding"]==3,vars.demo[var_id]]
    cat(paste(vars.demo[var_id], " within TumorBudding 1:\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.i.tumorbudding.1, na.rm = T), "(",
              min(data.i.tumorbudding.1, na.rm = T), ",",
              max(data.i.tumorbudding.1, na.rm = T), ")", sep = ""))
    cat("\n\n")
    cat(paste(vars.demo[var_id], " within TumorBudding 2:\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.i.tumorbudding.2, na.rm = T), "(",
              min(data.i.tumorbudding.2, na.rm = T), ",",
              max(data.i.tumorbudding.2, na.rm = T), ")", sep = ""))
    cat("\n\n")
    cat(paste(vars.demo[var_id], " within TumorBudding 3:\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.i.tumorbudding.3, na.rm = T), "(",
              min(data.i.tumorbudding.3, na.rm = T), ",",
              max(data.i.tumorbudding.3, na.rm = T), ")", sep = ""))
    cat("\n\n")
  }


}
```

```
## Age:
##    <50   >=75 50-74
##     27     40    133
##
##    <50   >=75 50-74
##   13.5   20.0   66.5
##
##
## Age X  TumorBudding Table (number):
##            TumorBudding
## Age          1   2   3
##    <50      13   4  10
##    >=75     22   8  10
##    50-74    62  24  47
## Age X TumorBudding  Table  (percentage):
##            TumorBudding
## Age            1      2      3
##    <50      13.4  11.1  14.9
##    >=75     22.7  22.2  14.9
##    50-74    63.9  66.7  70.1
## Fisher exact test p-value:0.7797
##
## Gender:
## Female   Male
##    102     98
##
## Female   Male
##     51     49
##
## Gender X TumorBudding Table (number):
```

```
##              TumorBudding
## Gender       1    2    3
##    Female 50 15 37
##    Male     47 21  30
## Gender X TumorBudding Table (percentage):
##              TumorBudding
## Gender          1       2       3
##    Female 51.5 41.7 55.2
##    Male     48.5 58.3  44.8
## Fisher exact test p-value:0.4367
##
## Race:
##    Black  Chinese  Unknown     White
##          17             2             1         180
##
##    Black  Chinese  Unknown     White
##        8.5         1.0         0.5        90.0
##
## Race X TumorBudding Table (number):
##                TumorBudding
## Race           1    2    3
##    Black       9    4    4
##    Chinese    1    0    1
##    Unknown  1    0    0
##    White      86 32  62
## Race X TumorBudding Table (percentage):
##                TumorBudding
## Race              1       2       3
##    Black       9.3  11.1    6.0
##    Chinese   1.0    0.0    1.5
##    Unknown  1.0    0.0    0.0
##    White      88.7 88.9  92.5
## Fisher exact test p-value:0.9108
##
## Stage:
##   1    2    3
## 57 74 69
##
##      1       2       3
## 28.5 37.0 34.5
##
## Stage X TumorBudding Table (number):
##          TumorBudding
## Stage    1    2    3
##        1  37  11    9
##        2 42 13 19
##        3 18 12 39
## Stage X TumorBudding Table (percentage):
##          TumorBudding
## Stage       1       2       3
##        1 38.1 30.6 13.4
##        2 43.3 36.1 28.4
##        3 18.6 33.3 58.2
## Fisher exact test p-value:0
```

```
##
## TumorLocation:
##      cecum noncecum
##         37       163
##
##      cecum noncecum
##       18.5      81.5
##
## TumorLocation X TumorBudding Table (number):
##                      TumorBudding
## TumorLocation    1   2   3
##         cecum      12   8  17
##         noncecum 85 28 50
## TumorLocation X TumorBudding Table (percentage):
##                      TumorBudding
## TumorLocation       1      2      3
##         cecum      12.4 22.2  25.4
##         noncecum 87.6 77.8 74.6
## Fisher exact test p-value:0.0812
##
## BMI:
## nonobese      Obese
##         136          64
##
## nonobese      Obese
##          68          32
##
## BMI X TumorBudding Table (number):
##                 TumorBudding
## BMI              1   2   3
##    nonobese 70 29 37
##    Obese      27   7  30
## BMI X TumorBudding Table (percentage):
##                 TumorBudding
## BMI                 1      2      3
##    nonobese 72.2 80.6 55.2
##    Obese      27.8 19.4  44.8
## Fisher exact test p-value:0.0157
##
## Desmoplasia:
##    1    2    3
## 124   28   48
##
##  1   2   3
## 62 14 24
##
## Desmoplasia X TumorBudding Table (number):
##                   TumorBudding
## Desmoplasia   1   2   3
##                 1 48 27 49
##                 2  15   5   8
##                 3  34   4  10
## Desmoplasia X TumorBudding Table (percentage):
##                   TumorBudding
```

```
## Desmoplasia       1       2       3
##                1 49.5 75.0 73.1
##                2 15.5 13.9 11.9
##                3 35.1 11.1 14.9
## Fisher exact test p-value:0.0049
##
## TumorClusters:
##    1    2    3
## 100   36   64
##
##  1   2   3
## 50 18 32
##
## TumorClusters X TumorBudding Table (number):
##                    TumorBudding
## TumorClusters    1   2   3
##                1 74   9  17
##                2 11 10 15
##                3 12 17 35
## TumorClusters X TumorBudding Table (percentage):
##                    TumorBudding
## TumorClusters       1       2       3
##                1 76.3 25.0 25.4
##                2 11.3 27.8 22.4
##                3 12.4 47.2 52.2
## Fisher exact test p-value:0
##
## TumorBorder:
## median(min,Max): 60(0,100)
## ANOVA p-value:0
##
## TumorBorder within TumorBudding 1:
## median(min,Max): 35(0,90)
##
## TumorBorder within TumorBudding 2:
## median(min,Max): 65(0,90)
##
## TumorBorder within TumorBudding 3:
## median(min,Max): 85(5,100)
##
## TumorStromaRatio:
## median(min,Max): 60(10,90)
## ANOVA p-value:0.0029
##
## TumorStromaRatio within TumorBudding 1:
## median(min,Max): 70(10,90)
##
## TumorStromaRatio within TumorBudding 2:
## median(min,Max): 60(10,90)
##
## TumorStromaRatio within TumorBudding 3:
## median(min,Max): 60(10,90)
##
## KMScore:
```

```
## median(min,Max): 1(0,3)
## ANOVA p-value:0.3908
##
## KMScore within TumorBudding 1:
## median(min,Max): 1(0,3)
##
## KMScore within TumorBudding 2:
## median(min,Max): 1(0,3)
##
## KMScore within TumorBudding 3:
## median(min,Max): 1(0,3)
##
## Necrosis:
## median(min,Max):  10(0,60)
## ANOVA p-value:0.0388
##
## Necrosis within TumorBudding 1:
## median(min,Max): 10(0,60)
##
## Necrosis within TumorBudding 2:
## median(min,Max): 10(0,50)
##
## Necrosis within TumorBudding 3:
## median(min,Max): 10(0,60)
```

2. Associations between tumor budding and clinical/histological features (Table 2)

As tumor budding is ordinal (low, intermediate, high), we utilized the class of cumulative logit models. A popular model in the class is the proportional odds model. To examine the applicability of the proportional odds model to our data, we first utilized a fully nonproportional odds model for tumor budding with BMI, age, gender, race, TNM stage, tumor location, Appalachian status, poorly differentiated tumor clusters, desmoplasia, infiltrative tumor border, tumor to stroma ratio, KM inflammatory score and tumor necrosis as explanatory variables. Based on this model, we assessed the proportional odds assumption for each of the explanatory variables by testing whether the model parameters for an explanatory variable across the logits are the same based on a Wald test. Note that Patients with Chinese or unknown race (n=3) were excluded in this analysis.

```
library(ordinal)
nonPO.fit <- clm(TumorBudding ~ 1,
                 nominal = ~  BMI + Age + Gender + Race + Stage + TumorLocation  +
                 AppalachianStatus + TumorClusters + Desmoplasia + TumorBorder +
                 TumorStromaRatio + KMScore + Necrosis, data=data.use)
variables = c("BMI", "Age", "Gender", "Race", "Stage", "TumorLocation",
              "AppalachianStatus", "TumorClusters", "Desmoplasia", "TumorBorder",
              "TumorStromaRatio", "KMScore", "Necrosis")
PO.pval = matrix(nrow=length(variables),ncol=1)
rownames(PO.pval) = variables; colnames(PO.pval) = "p-value"
for (i in 1:length(variables)){
  PO.pval[i] = check_PO(vcov = vcov(nonPO.fit),
                        coef.beta = coef(nonPO.fit),
                        coef.name =  variables[i])
}
print(PO.pval)
```

```
## BMI                  0.1333
## Age                  0.7257
## Gender               0.0490
## Race                 0.6327
## Stage                0.0180
## TumorLocation        0.8323
## AppalachianStatus    0.0821
## TumorClusters        0.0921
## Desmoplasia          0.1779
## TumorBorder          0.6020
## TumorStromaRatio     0.6620
## KMScore              0.0293
## Necrosis             0.0492 ##   p-value
```

Based on the above test, the proportional odds assumption appeared to hold for most explanatory variables (non-significant p-values) except for gender, TNM stage, KM inflammatory score and tumor necrosis (significant p-values). Therefore, we fitted the following partial proportional odds logistic model, which assumed proportional odds effects for BMI, age, race, tumor location, Appalachian status, poorly differentiated tumor clusters, desmoplasia, infiltrative tumor border, and tumor to stroma ratio, and non-proportional odds effects for gender, TNM stage, KM inflammatory score and tumor necrosis.

```
partialPO.fit <- clm(TumorBudding ~ BMI + Age + Race + TumorLocation +
                       AppalachianStatus + TumorClusters + Desmoplasia + TumorBorder +
                       TumorStromaRatio,
                       nominal = ~ Gender + Stage + KMScore + Necrosis, data=data.use)
print(summary(partialPO.fit))
```

```
## formula:
## TumorBudding ~ BMI + Age + Race + TumorLocation + AppalachianStatus +
TumorClusters + Desmoplasia + TumorBorder + TumorStromaRatio
## nominal: ~Gender + Stage + KMScore + Necrosis
## data: data.use
##
## link threshold nobs logLik AIC niter max.grad cond.H
## logit flexible 194 -144.09 336.19 6(0) 2.89e-11 9.6e+05
##
## Coefficients:
## Estimate Std. Error z value Pr(>|z|)
## BMIObese 1.447263 0.397438 3.641 0.000271 ***
## Age>=75 -0.117708 0.662995 -0.178 0.859085
## Age50-74 0.388058 0.566052 0.686 0.492996
## RaceWhite -0.124392 0.660396 -0.188 0.850594
## TumorLocationcecum 0.934632 0.435025 2.148 0.031678 *
##  AppalachianStatusApp  0.433238  0.344425  1.258  0.208443
##  TumorClusters2  2.212302  0.491289  4.503  6.70e-06  ***
##  TumorClusters3  1.628388  0.404964  4.021  5.79e-05  ***
## Desmoplasia2 -1.065458 0.483172 -2.205 0.027445 *
## Desmoplasia3 -0.660757 0.474347 -1.393 0.163625
## TumorBorder 0.026863 0.006605 4.067 4.76e-05 ***
## TumorStromaRatio 0.003584 0.008671 0.413 0.679318
## ---
## Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Threshold coefficients:
```

```
## Estimate Std. Error z value
##  1|2.(Intercept)  3.761588  1.250816  3.007
##  2|3.(Intercept)  4.682715  1.293688  3.620
## 1|2.GenderMale -0.534194 0.390993 -1.366
## 2|3.GenderMale 0.096174 0.397799 0.242
## 1|2.Stage2 0.181677 0.495803 0.366
##  2|3.Stage2  -0.020261  0.568748 -0.036
##  1|2.Stage3  -1.068812  0.545701 -1.959
##  2|3.Stage3  -1.199386  0.583528 -2.055
## 1|2.KMScore -0.086169 0.272475 -0.316
## 2|3.KMScore 0.247880 0.339649 0.730
## 1|2.Necrosis 0.009187 0.017091 0.538
## 2|3.Necrosis -0.007203 0.017016 -0.423
## (3 observations deleted due to missingness)
```

We performed the Pulkstenis-Robinson goodness-of-fit tests, which supported the adequacy of the model.

```
library(generalhoslem)
```

```
## Loading required package:  reshape
## Loading required package: MASS
```

```
pulkrob.chisq(partialPO.fit, variables)
```

```
##
## Pulkstenis-Robinson chi-squared test
##
## data: formula: TumorBudding ~ BMI + Age + Race +
TumorLocation + AppalachianStatus + formula: TumorClusters
+ Desmoplasia + TumorBorder + TumorStromaRatio
## X-squared = 365.91, df = 370, p-value = 0.5503
```

```
pulkrob.deviance(partialPO.fit,   variables)
```

```
##
## Pulkstenis-Robinson deviance test
##
## data: formula: TumorBudding ~ BMI + Age + Race +
TumorLocation + AppalachianStatus + formula: TumorClusters
+ Desmoplasia + TumorBorder + TumorStromaRatio
## Deviance-squared = 288.19, df = 370, p-value = 0.9994
```

Based on this partial proportional odds logistic model, we performed a Wald test to assess the association between tumor budding and each of the clinical/histological features.

```
pvalue = NULL
for (i in 1:length(variables)){

  if (variables[i] %in% c("Gender", "Stage", "KMScore", "Necrosis")){
    pv.ci.fit <- Wald_pvalue_CI(vcov = vcov(partialPO.fit),
                                coef.beta = coef(partialPO.fit),
                                coef.name = variables[i], nominal = T)
  } else{
    pv.ci.fit <- Wald_pvalue_CI(vcov =  vcov(partialPO.fit),
                                coef.beta = coef(partialPO.fit),
                                coef.name =  variables[i])
  }
```

```
print(variables[i])
print(pv.ci.fit)
pvalue[i] = pv.ci.fit$p_value
}
```

```
## [1] "BMI"
## $p_value
## [1] 3e-04
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]            4.25     1.95     9.26
##
## [1] "Age"
## $p_value
## [1] 0.4444
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]            0.89     0.24     3.26
## [2,]            1.47     0.49     4.47
##
## [1] "Gender"
## $p_value
## [1] 0.1969
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]            1.71     0.79     3.67
## [2,]            0.91     0.42     1.98
##
## [1] "Race"
## $p_value
## [1] 0.8506
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]            0.88     0.24     3.22
##
## [1] "Stage"
## $p_value
## [1] 0.0376
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]            0.83     0.32     2.20
## [2,]            1.02     0.33     3.11
## [3,]            2.91     1.00     8.49
## [4,]            3.32     1.06    10.41
##
## [1] "TumorLocation"
## $p_value
## [1] 0.0317
##
```

```
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]           2.55     1.09     5.97
##
## [1] "AppalachianStatus"
## $p_value
## [1] 0.2084
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]           1.54     0.79     3.03
##
## [1] "TumorClusters"
## $p_value
## [1] 0
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]           9.14     3.49    23.93
## [2,]           5.10     2.30    11.27
##
## [1] "Desmoplasia"
## $p_value
## [1] 0.051
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]           0.34     0.13     0.89
## [2,]           0.52     0.20     1.31
##
## [1] "TumorBorder"
## $p_value
## [1] 0
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]           1.03     1.01     1.04
##
## [1] "TumorStromaRatio"
## $p_value
## [1] 0.6793
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]              1     0.99     1.02
##
## [1] "KMScore"
## $p_value
## [1] 0.5809
##
## $estimation
##       point_estimate CI_lower CI_upper
## [1,]           1.09     0.64     1.86
## [2,]           0.78     0.40     1.52
```

```
##
## [1] "Necrosis"
## $p_value
## [1] 0.6264
##
## $estimation
##      point_estimate CI_lower CI_upper
## [1,]           0.99     0.96     1.02
## [2,]           1.01     0.97     1.04
```

```
names(pvalue) = variables
print(pvalue)
```

```
##        BMI        Age     Gender       Race
##     0.0003     0.4444     0.1969     0.8506
##      Stage TumorLocation AppalachianStatus TumorClusters
##     0.0376     0.0317     0.2084     0.0000
##  Desmoplasia TumorBorder TumorStromaRatio KMScore
##     0.0510     0.0000     0.6793     0.5809
##  Necrosis
##     0.6264
```

Based on the above analysis, we identified significant associations between tumor budding and BMI, stage, tumor location, poorly differentiated tumor clusters and infiltrative tumor border. Note that for BMI, tumor location, poorly differentiated tumor clusters and infiltrative tumor border, a single odds ratio was reported because the proportional odds assumption held. For TNM stage, two separate odds ratios were reported for each level of stage because the proportional odds assumption was not satisfied for this variable.

3. Association between tumor budding and survival time (Figure 1 and Table 3)

We first used Kaplan-Meier curves and logrank test to assess the association between tumor budding and survival time.

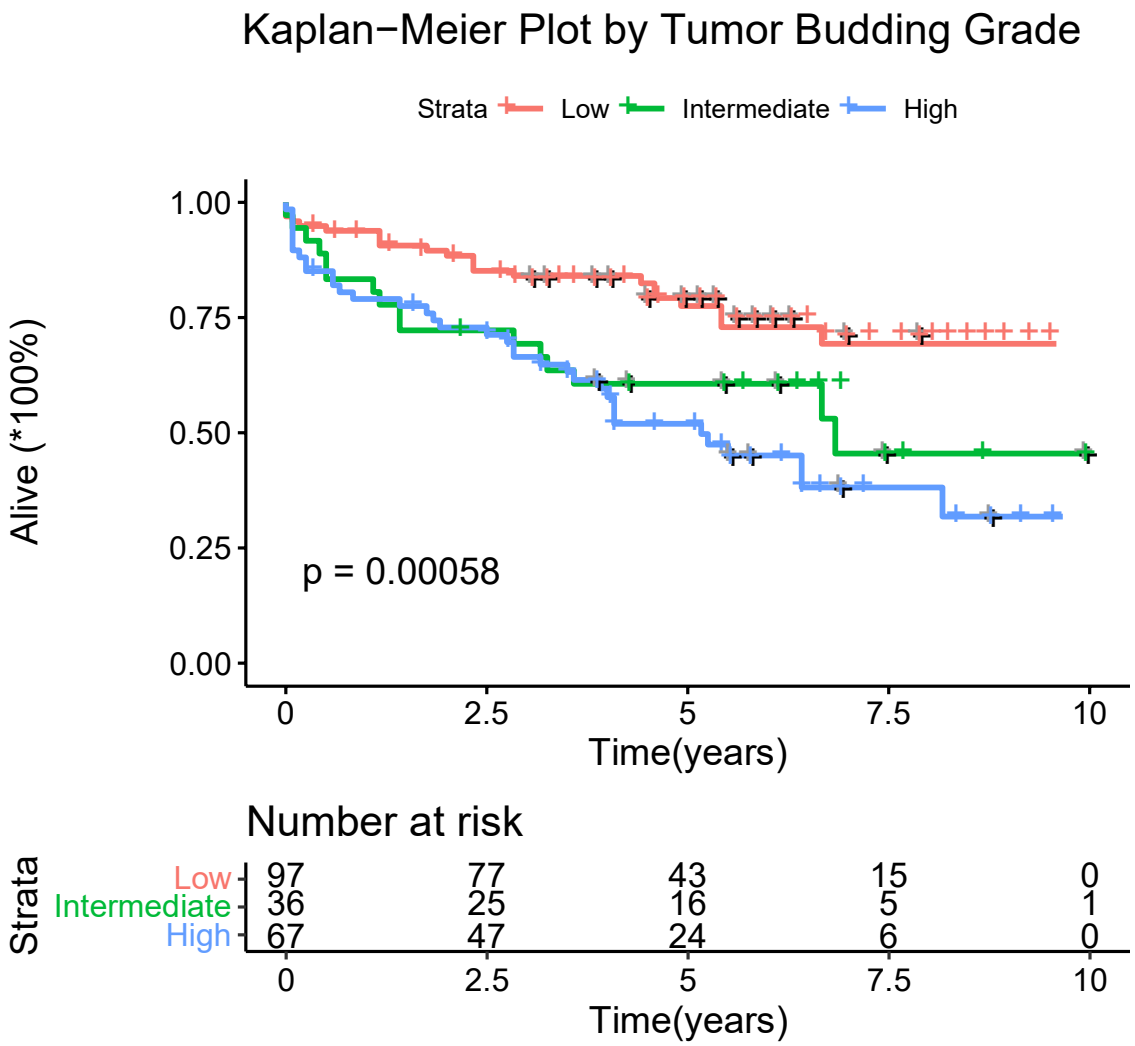
```
library(survival)
library(survminer)
```

```
## Loading required package: ggplot2
## Loading required package: ggpubr
```

```
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data.use1)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data.use1)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time across tumor budding groups:",
    round(thispvalue,digits=5), "\n\n")
```

```
## Logrank P for comparing survival time across tumor budding groups: 0.00058
```

```
ggsurvplot(fit, data=data.use1, risk.table = TRUE, pval=T,
           legend.labs=c("Low", "Intermediate", "High"),
           title="Kaplan-Meier Plot by Tumor Budding Grade",
           xlab="Time(years)", ylab="Alive (*100%)")
```



Pairwise comparisons of survival time between two levels of tumor budding were also performed using logrank tests.

```
data12 <- data.use1[data.use1$TumorBudding==1 | data.use1$TumorBudding==2, ]
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data12)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data12)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time between intermediate vs low tumor budding:",
    round(thispvalue,digits=4), "\n\n")
```

```
## Logrank P for comparing survival time between
intermediate vs low tumor budding: 0.0232
```

```
data13 <- data.use1[data.use1$TumorBudding==1 | data.use1$TumorBudding==3, ]
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data13)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data13)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time between high vs low tumor budding:",
    round(thispvalue,digits=4), "\n\n")
```

```
## Logrank P for comparing survival time between high vs
low tumor budding: 1e-04
```

```
data23 <- data.use1[data.use1$TumorBudding==2 | data.use1$TumorBudding==3, ]
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data23)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data23)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time between high vs intermediate tumor budding:",
    round(thispvalue,digits=4), "\n\n")
```

```
## Logrank P for comparing survival time between high vs
intermediate tumor budding: 0.3513
```

We next used a proportional hazards model to study the association between tumor budding and survival time with adjustment for BMI, age, gender, race, TNM stage, tumor location, and Appalachian status. Note that Patients with Chinese or unknown race (n=3) were excluded in this analysis.

```
fit.cox <- coxph(Surv(Survtime, Status) ~ TumorBudding + BMI + Age + Gender + Race + Stage +
                   TumorLocation + AppalachianStatus, data=data.use)
```

We checked the proportional hazards assumption based on the method proposed by Grambsch and Therneau (1994). As shown below, the assumption appeared to hold for all explanatory variables (non-significant p-values).

```
cox.zph(fit.cox)
```

```
##                      chisq  df    p
## TumorBudding       0.64905   2 0.72
## BMI                0.52991   1 0.47
## Age                4.12511   2 0.13
## Gender             0.00616   1 0.94
## Race               2.15933   1 0.14
## Stage              0.55600   2 0.76
## TumorLocation      0.10567   1 0.75
## AppalachianStatus  0.48316   1 0.49
## GLOBAL             8.42014  11 0.68
```

We also performed the goodness-of-fit test proposed by May and Hosmer (2004), which supported the adequacy of the model.

```
library(survMisc)
```

```
##
## Attaching package: 'survMisc'
## The following object is masked from 'package:ggplot2':
##
##     autoplot
```

```
print(gof(fit.cox), maxCol=20)
```

```
## $groups
## n e exp z p
## 1: <multi-column> 20 19.37962 0.14092477 0.8879294
## 2: <multi-column> 53 53.62038 -0.08472176 0.9324826
##
## $lrTest
## Analysis of Deviance Table
## Cox model: response is Surv(Survtime, Status)
## Model 1: ~ TumorBudding + BMI + Age + Gender + Race +
Stage + TumorLocation + AppalachianStatus
```

```
## Model 2: ~ TumorBudding + BMI + Age + Gender + Race +
Stage + TumorLocation + AppalachianStatus + indicG
## loglik Chisq Df P(>|Chi|)
## 1 -337.11
## 2 -337.08 0.0694 1 0.7922
```

Based on this model, we identified a significant association between tumor budding and survival time.

```
variables = c("TumorBudding", "BMI", "Age", "Gender", "Race", "Stage", "TumorLocation",
              "AppalachianStatus")
for (i in 1:length(variables)){
  cox.pvalue.ci <- Wald_pvalue_CI(vcov = vcov(fit.cox),
                                  coef.beta = summary(fit.cox)$coefficients[ ,1],
                                  coef.name = variables[i])
  print(paste("For ", variables[i]))
  print(cox.pvalue.ci)
}
```

```
## [1] "For  TumorBudding"
## $p_value
## [1] 0.0045
##
## $estimation
##      point_estimate CI_lower CI_upper
## [1,]           2.20     1.11     4.35
## [2,]           2.67     1.45     4.90
##
## [1] "For  BMI"
## $p_value
## [1] 0.6451
## $estimation
##      point_estimate CI_lower CI_upper
## [1,]           1.13     0.66     1.93
##
## [1] "For  Age"
## $p_value
## [1] 1e-04
##
## $estimation
##      point_estimate CI_lower CI_upper
## [1,]           3.89     1.59     9.50
## [2,]           1.30     0.57     2.98
##
## [1] "For  Gender"
## $p_value
## [1] 0.5061
##
## $estimation
##      point_estimate CI_lower CI_upper
## [1,]           0.85     0.52     1.38
##
## [1] "For  Race"
## $p_value
## [1] 0.3039
```

```
##
## $estimation
##        point_estimate CI_lower CI_upper
##  [1,]           1.75      0.6      5.1
##
##  [1]  "For    Stage"
##  $p_value
## [1] 0.3668
##
## $estimation
##        point_estimate CI_lower CI_upper
##  [1,]           1.27     0.65     2.45
##  [2,]           1.61     0.82     3.14
##
##  [1]  "For    TumorLocation"
##  $p_value
## [1] 0.9438
##
## $estimation
##        point_estimate CI_lower CI_upper
##  [1,]           1.02     0.56     1.88
##
##  [1]  "For    AppalachianStatus"
##  $p_value
## [1] 0.6932
##
## $estimation
##        point_estimate CI_lower CI_upper
##  [1,]           1.11     0.67     1.84
```

References

P. Grambsch and T. Therneau (1994), Proportional hazards tests and diagnostics based on weighted residuals. Biometrika, 81, 515-26.

May S, Hosmer DW 2004. A cautionary note on the use of the Gronnesby and Borgan goodness-of-fit test for the Cox proportional hazards model. Lifetime Data Analysis 10(3):283–91.

B. Complete R Code

```r
# Data processing
data.all <- read.delim(file = "2-27-20 Tumor Bud BMI Final Data Sheet Deidentified_use.txt",
                       header = T, stringsAsFactors = F)
data.all$TumorBorder <- as.numeric(data.all$Invasive.Front.....)
data.all$Desmoplasia <- data.all$Desmoplasia..Ueno.Score.
data.all$Desmoplasia[data.all$Desmoplasia..Ueno.Score.%in%c("4", "3,4", "4,2")] <- 3
data.all$Desmoplasia[data.all$Desmoplasia==""] = "Unknown"

data.all <- data.all[!data.all$beststagegroup%in%c("Stage 0", "Stage IV", "Stage Unknown"), ]

data.all <- data.all[(data.all$Tumor.Buds.Score..Ueno.Scheme.!=0) &
                       !is.na(data.all$Tumor.Buds.Score..Ueno.Scheme.), ]

data.all$Age <- NA
```

```r
data.all$Age[data.all$diagage<50] <- "<50"
data.all$Age[data.all$diagage>=50 & data.all$diagage<=74] <- "50-74"
data.all$Age[data.all$diagage>=75] <- ">=75"

data.all$Gender <- data.all$gender

data.all$Race <- data.all$race

data.all$AppalachianStatus <- data.all$appalachia_status

data.all$Stage <- data.all$beststagegroup
data.all$Stage[data.all$beststagegroup=="Stage I"] <- "1"
data.all$Stage[data.all$beststagegroup%in%c("Stage IIB", "Stage IIA")] <- "2"
data.all$Stage[data.all$beststagegroup%in%c("Stage IIIA", "Stage IIIB", "Stage IIIC")] <- "3"

data.all$Stage[data.all$beststagegroup.regrp=="Stage Unknown"] <- NA

data.all$Location.QC[data.all$Location.QC=="10"] <- "1"
data.all$Location.QC[data.all$Location.QC=="2,3"] <- "2"
data.all$TumorLocation <- NA
data.all$TumorLocation[data.all$Location.QC=="1"] <- "cecum"
data.all$TumorLocation[data.all$Location.QC!="1" & data.all$Location.QC!=""] <- "noncecum"
data.all$TumorLocation[data.all$Location.QC==""] <- "Unknown"

data.all$BMI.regrp <- NA
data.all$BMI.regrp[data.all$BMI < 30] <- "nonobese"
data.all$BMI.regrp[data.all$BMI >= 30] <- "Obese"
data.all$BMI <- data.all$BMI.regrp

data.all$TumorClusters <- data.all$PD.tumor.clusters

data.all$Survtime <- data.all$survmonths/12
data.all$Status <- NA
data.all$Status[grep("Alive", data.all$vital_status)] <- 0
data.all$Status[grep("Dead", data.all$vital_status)] <- 1

data.all$TumorBudding <- data.all$Tumor.Buds.Score..Ueno.Scheme.

data.all$TumorStromaRatio = data.all$Tumor.Stroma.Ratio....

data.all$KMScore = data.all$K.M.Score

data.all$Necrosis = data.all$Necrosis....

# demographic and clinical characteristics (Table 1)
vars.demo <- c("Age", "Gender", "Race", "Stage", "TumorLocation",
               "BMI", "Desmoplasia", "TumorClusters", "TumorBorder", "TumorStromaRatio",
               "KMScore", "Necrosis")
vars.type <- c("C", "C", "C", "C", "C",
               "C", "C", "C", "N", "N", "N", "N")
```

```
for (var_id in 1:length(vars.demo)) {

  if(vars.type[var_id]=="C"){
    table.self <- table(data.all[ ,vars.demo[var_id]])
    table.cross <- table(data.all[ ,c(vars.demo[var_id], "TumorBudding")])
    percent.cross <- round(t(t(table.cross) / colSums(table.cross))*100,digits=1)
    fisher.result <- fisher.test(table.cross)
    cat(paste(vars.demo[var_id], ":", sep = ""))
    print(table.self)
    print(round(table.self/sum(table.self)*100, digits=1))
    cat("\n")
    cat(paste(vars.demo[var_id], " X ", "TumorBudding Table (number):", "\n", sep = ""))
    print(table.cross)
    cat(paste(vars.demo[var_id], " X ", "TumorBudding Table (percentage):", "\n", sep = ""))
    print(percent.cross)
    cat("Fisher exact test p-value:")
    cat(round(fisher.result$p.value,digits=4), "\n\n")
  } else{
    cat(paste(vars.demo[var_id], ":\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.all[ ,vars.demo[var_id]], na.rm = T), "(",
              min(data.all[ ,vars.demo[var_id]], na.rm = T), ",",
              max(data.all[ ,vars.demo[var_id]], na.rm = T), ")", sep = ""))
    fit.anova <- aov(data.all[ ,vars.demo[var_id]] ~ data.all[ ,"TumorBudding"])
    p.anova <- round(summary(fit.anova)[[1]]$Pr[1],digits=4)
    cat("\n")
    cat("ANOVA p-value:")
    cat(p.anova, "\n\n")
    data.i.tumorbudding.1 <- data.all[data.all[ ,"TumorBudding"]==1,vars.demo[var_id]]
    data.i.tumorbudding.2 <- data.all[data.all[ ,"TumorBudding"]==2,vars.demo[var_id]]
    data.i.tumorbudding.3 <- data.all[data.all[ ,"TumorBudding"]==3,vars.demo[var_id]]
    cat(paste(vars.demo[var_id], " within TumorBudding 1:\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.i.tumorbudding.1, na.rm = T), "(",
              min(data.i.tumorbudding.1, na.rm = T), ",",
              max(data.i.tumorbudding.1, na.rm = T), ")", sep = ""))
    cat("\n\n")
    cat(paste(vars.demo[var_id], " within TumorBudding 2:\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.i.tumorbudding.2, na.rm = T), "(",
              min(data.i.tumorbudding.2, na.rm = T), ",",
              max(data.i.tumorbudding.2, na.rm = T), ")", sep = ""))
    cat("\n\n")
    cat(paste(vars.demo[var_id], " within TumorBudding 3:\n", sep = ""))
    cat("median(min,Max): ")
    cat(paste(median(data.i.tumorbudding.3, na.rm = T), "(",
              min(data.i.tumorbudding.3, na.rm = T), ",",
              max(data.i.tumorbudding.3, na.rm = T), ")", sep = ""))
    cat("\n\n")
  }
}

data.TumorBuddingFiltered <- data.all
```

```
data.TumorBuddingFiltered$TumorBudding <- factor(data.TumorBuddingFiltered$TumorBudding)
data.TumorBuddingFiltered$BMI <- factor(data.TumorBuddingFiltered$BMI,
                                        levels = c("nonobese", "Obese"))
data.TumorBuddingFiltered[ ,"TumorClusters"] <- factor(data.TumorBuddingFiltered[ ,"TumorClusters"],
                                                       levels = c(1,2,3))
data.TumorBuddingFiltered[ ,"Gender"] <- factor(data.TumorBuddingFiltered[ ,"Gender"],
                                                levels = c("Female", "Male"))
data.TumorBuddingFiltered[ ,"TumorLocation"] <- factor(data.TumorBuddingFiltered[ ,"TumorLocation"],
                                                       levels = c("cecum", "noncecum"))
data.TumorBuddingFiltered$TumorLocation = relevel(data.TumorBuddingFiltered$TumorLocation,
                                                  ref="noncecum")
data.TumorBuddingFiltered[ ,"Stage"] <- factor(data.TumorBuddingFiltered[ ,"Stage"],
                                               levels = c("1", "2", "3"))
data.TumorBuddingFiltered[ ,"Desmoplasia"] <- factor(data.TumorBuddingFiltered[ ,"Desmoplasia"],
                                                     levels = c(1,2,3))
data.TumorBuddingFiltered$AppalachianStatus[data.TumorBuddingFiltered$AppalachianStatus==
                                              "Appalachian county"] <- "App"
data.TumorBuddingFiltered$AppalachianStatus[data.TumorBuddingFiltered$AppalachianStatus
                                            =="not Appalachian county"] <- "nonApp"
data.TumorBuddingFiltered$AppalachianStatus <- factor(data.TumorBuddingFiltered$AppalachianStatus,
                                                      levels = c("nonApp", "App"))
data.use1 = data.TumorBuddingFiltered
data.TumorBuddingFiltered = data.TumorBuddingFiltered[(data.TumorBuddingFiltered$Race != "Unknown") &
                                                        (data.TumorBuddingFiltered$Race!="Chinese"), ]
data.TumorBuddingFiltered[ ,"Race"] <- factor(data.TumorBuddingFiltered[ ,"Race"])

clinical.factors <- c("BMI", "Age", "Gender", "Race", "Stage", "TumorLocation","AppalachianStatus")

histological.factors <- c("TumorClusters", "Desmoplasia", "TumorBorder",
                          "TumorStromaRatio", "KMScore", "Necrosis")

data.use <- data.TumorBuddingFiltered[,c("TumorBudding",clinical.factors,
                                         histological.factors, "Survtime", "Status")]

# Associations between  tumor budding  and clinical/histological features (Table 2)
# Auxiliary functions
Wald_pvalue_CI <- function(vcov, coef.beta, coef.name, nominal=FALSE)
{
  vcov.use = vcov[grep(coef.name, row.names(vcov)),grep(coef.name, colnames(vcov))]
  if(class(coef.beta)[1]=="numeric") {coef.beta.use =
    coef.beta[grep(coef.name, names(coef.beta))];}
  if(class(coef.beta)[1]=="matrix") {coef.beta.use =
    coef.beta[,grep(coef.name, colnames(coef.beta))];}

  if(!is.null(dim(vcov.use)))
  {
    A.m = diag(dim(vcov.use)[1])
    beta.m = matrix(coef.beta.use, ncol = 1)
    val.chisqr.m = t(A.m%*%beta.m)%*%solve(t(A.m)%*%vcov.use%*%A.m)%*%(A.m%*%beta.m)

    pval = round(as.numeric(1-pchisq(val.chisqr.m, df=dim(vcov.use)[1])), digits=4)
```

```
    CI = matrix(NA, nrow = dim(vcov.use)[1], ncol = 3)
    for(III in 1:dim(vcov.use)[1])
    {
      if (nominal){
        CI[III, 1] <- exp((-1)*beta.m[III,1])
        CI[III, 2] <- exp((-1)*beta.m[III,1] - abs(qnorm(0.025))*sqrt(vcov.use[III,III]))
        CI[III, 3] <- exp((-1)*beta.m[III,1] + abs(qnorm(0.025))*sqrt(vcov.use[III,III]))
      } else{
        CI[III, 1] <- exp(beta.m[III,1])
        CI[III, 2] <- exp(beta.m[III,1] - abs(qnorm(0.025))*sqrt(vcov.use[III,III]))
        CI[III, 3] <- exp(beta.m[III,1] + abs(qnorm(0.025))*sqrt(vcov.use[III,III]))
      }
    }
    CI = round(CI, digits=2)
    colnames(CI) <- c("point_estimate", "CI_lower", "CI_upper")
  }

  if(is.null(dim(vcov.use)))
  {
    val.chisqr.s = (coef.beta.use)^2/vcov.use
    pval = round(as.numeric(1-pchisq(val.chisqr.s, df=1)), digits=4)

    CI = matrix(NA, nrow = 1, ncol = 3)
    CI[1, 1] = exp(coef.beta.use)
    CI[1, 2] = exp(coef.beta.use - abs(qnorm(0.025))*sqrt(vcov.use))
    CI[1, 3] = exp(coef.beta.use + abs(qnorm(0.025))*sqrt(vcov.use))
    CI = round(CI, digits=2)
    colnames(CI) = c("point_estimate", "CI_lower", "CI_upper")
  }

  list.return = list()
  list.return[[1]] = pval
  list.return[[2]] = CI
  names(list.return) = c("p_value", "estimation")
  return(list.return)
}

check_PO <- function(vcov, coef.beta, coef.name)
{
  vcov.use = vcov[grep(coef.name, row.names(vcov)),grep(coef.name, colnames(vcov))]
  if(class(coef.beta)[1]=="numeric") {coef.beta.use =
    coef.beta[grep(coef.name, names(coef.beta))];}
  if(class(coef.beta)[1]=="matrix") {coef.beta.use =
    coef.beta[,grep(coef.name, colnames(coef.beta))];}

  if(!is.null(dim(vcov.use)))
  {
    dd = dim(vcov.use)[1]
    A.m = matrix(0, nrow=dd-1, ncol=dd)
    for (i in 1:(dd-1)){A.m[i,i] = 1; A.m[i,i+1] = -1}
    beta.m = matrix(coef.beta.use, ncol = 1)
    val.chisqr.m = t(A.m%*%beta.m)%*%solve(A.m%*%vcov.use%*%t(A.m))%*%(A.m%*%beta.m)
```

```
    pval = round(as.numeric(1-pchisq(val.chisqr.m, df=dd-1)),digits=4)
  }

  if(is.null(dim(vcov.use)))
  {
    pval = NA
  }
  return(pval)
}


library(ordinal)
nonPO.fit <- clm(TumorBudding ~ 1,
                 nominal = ~  BMI + Age + Gender + Race + Stage + TumorLocation  +
                 AppalachianStatus + TumorClusters + Desmoplasia + TumorBorder +
                 TumorStromaRatio + KMScore + Necrosis, data=data.use)
variables = c("BMI", "Age", "Gender", "Race", "Stage", "TumorLocation",
              "AppalachianStatus", "TumorClusters", "Desmoplasia", "TumorBorder",
              "TumorStromaRatio", "KMScore", "Necrosis")
PO.pval = matrix(nrow=length(variables),ncol=1)
rownames(PO.pval) = variables; colnames(PO.pval) = "p-value"
for (i in 1:length(variables)){
  PO.pval[i] = check_PO(vcov = vcov(nonPO.fit),
                        coef.beta = coef(nonPO.fit),
                        coef.name =  variables[i])
}

print(PO.pval)

partialPO.fit <- clm(TumorBudding ~ BMI + Age  + Race + TumorLocation  +
                     AppalachianStatus + TumorClusters + Desmoplasia + TumorBorder +
                     TumorStromaRatio,
                     nominal = ~  Gender + Stage + KMScore + Necrosis, data=data.use)
print(summary(partialPO.fit))

library(generalhoslem)
pulkrob.chisq(partialPO.fit, variables)
pulkrob.deviance(partialPO.fit, variables)

pvalue = NULL
for (i in 1:length(variables)){

  if (variables[i] %in% c("Gender", "Stage", "KMScore", "Necrosis")){
    pv.ci.fit <- Wald_pvalue_CI(vcov = vcov(partialPO.fit),
                                coef.beta = coef(partialPO.fit),
                                coef.name = variables[i], nominal = T)
  } else{
    pv.ci.fit <- Wald_pvalue_CI(vcov = vcov(partialPO.fit),
                                coef.beta = coef(partialPO.fit),
                                coef.name =  variables[i])
  }
print(variables[i])
print(pv.ci.fit)
```

```
pvalue[i] = pv.ci.fit$p_value
}
names(pvalue) = variables
print(pvalue)

# Association between tumor budding and survival time (Figure 1 and Table 3)
library(survival)
library(survminer)
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data.use1)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data.use1)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time across tumor budding groups:",
    round(thispvalue,digits=5), "\n\n")
setEPS()
postscript("figure2.eps")
ggsurvplot(fit, data=data.use1, risk.table = TRUE, pval=T,
           legend.labs=c("Low", "Intermediate", "High"),
           title="Kaplan-Meier Plot by Tumor Budding Grade",
           xlab="Time(years)", ylab="Alive (*100%)")
dev.off()

data12 <- data.use1[data.use1$TumorBudding==1 | data.use1$TumorBudding==2, ]
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data12)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data12)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time between intermediate vs low tumor budding:",
    round(thispvalue,digits=4), "\n\n")

data13 <- data.use1[data.use1$TumorBudding==1 | data.use1$TumorBudding==3, ]
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data13)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data13)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time between high vs low tumor budding:",
    round(thispvalue,digits=4), "\n\n")

data23 <- data.use1[data.use1$TumorBudding==2 | data.use1$TumorBudding==3, ]
fit <- survfit(Surv(Survtime, Status) ~ TumorBudding, data=data23)
temp <- survdiff(Surv(Survtime, Status) ~ TumorBudding, data=data23)
thispvalue <- (1 - pchisq(temp$chisq, length(temp$n) - 1))
cat("Logrank P for comparing survival time between high vs intermediate tumor budding:",
    round(thispvalue,digits=4), "\n\n")

fit.cox <- coxph(Surv(Survtime, Status) ~ TumorBudding + BMI + Age + Gender + Race + Stage +
                   TumorLocation + AppalachianStatus, data=data.use)

cox.zph(fit.cox)

library(survMisc)
print(gof(fit.cox), maxCol=20)

variables = c("TumorBudding", "BMI", "Age", "Gender", "Race", "Stage", "TumorLocation",
              "AppalachianStatus")
for (i in 1:length(variables)){
```

```
  cox.pvalue.ci <- Wald_pvalue_CI(vcov = vcov(fit.cox),
                                  coef.beta  =  summary(fit.cox)$coefficients[  ,1],
                                  coef.name = variables[i])
  print(paste("For ", variables[i]))
  print(cox.pvalue.ci)
}
```